

EFFECTS OF PREBIOTICS ON GROWTH PERFORMANCE, NUTRIENT
UTILIZATION AND THE GASTROINTESTINAL TRACT MICROBIAL
COMMUNITY OF HYBRID STRIPED BASS (*Morone chrysops* x *M. saxatilis*) AND
RED DRUM (*Sciaenops ocellatus*)

A Dissertation

by

GARY STEPHEN BURR

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2007

Major Subject: Wildlife and Fisheries Sciences

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ABSTRACT

Effects of Prebiotics on Growth Performance, Nutrient Utilization and Gastrointestinal
Tract Microbial Community of
Hybrid Striped Bass (*Morone chrysops* x *M. saxatilis*) and Red Drum (*Sciaenops
ocellatus*). (December 2007)

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A series of experiments examined the effects of four potential prebiotics--GroBiotic[®]-A (a mixture of partially autolyzed brewers yeast, dairy components and dried fermentation products), mannanoligosaccharide (MOS), galactooligosaccharide (GOS), and inulin/ fructooligosaccharide (FOS)--on the gastrointestinal (GI) tract's microbial community in hybrid striped bass and red drum. The first *in vitro* experiment applied denaturing gradient gel electrophoresis (DGGE) to examine responses of red drum GI tract microbiota to anaerobic incubation with brewers yeast, FOS, and GroBiotic[®]-A. Brewers yeast and GroBiotic[®]-A produced unique microbial communities compared to that associated with the basal diet. Volatile fatty acid (VFA) profiles did not differ among treatments, with acetate being the major fermentation product. A second *in vitro* experiment examined effects of GroBiotic[®]-A, MOS, GOS, and FOS on the GI tract microbiota of hybrid striped bass. None of the prebiotics altered

the culturable microbial community, but all tended to lower acetate production and increase butyrate production. A third experiment examined the effects of the four prebiotics fed to juvenile hybrid striped bass for 8 weeks. Growth, feed efficiency ratio (FER) and protein efficiency ratio (PER) were not affected by the different prebiotics, but the GI tract's microbial community was altered from that associated with the basal diet. The fourth experiment consisted of an 8-week feeding trial and one 6-week feeding trial in which the effects of GroBiotic[®]-A and FOS on growth performance and microbial community composition were compared for red drum living in independent tanks versus tanks with a shared water system. Neither the intestinal microbial community nor growth performance were significantly altered by the prebiotics in these trials; fish in independent and shared water tanks produced similar results. The final experiment examined the effects of GroBiotic[®]-A, FOS, MOS and GOS on nutrient and energy digestibility of sub-adult red drum fed diets containing fish meal and soybean meal. The prebiotics generally increased protein, organic matter, and energy digestibility, with the exception of FOS/inulin. Lipid digestibility was decreased by GOS, MOS and FOS. These studies are the first to establish that prebiotics can alter the GI tract microbial community of these fish and influence nutrient digestibility.

DEDICATION

To my wife and family

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CHAPTER I

INTRODUCTION: MICROBIAL ECOLOGY OF THE GASTROINTESTINAL TRACT OF FISH AND THE POTENTIAL APPLICATION OF PREBIOTICS AND PROBIOTICS IN FINFISH AQUACULTURE*

Synopsis

Aquaculture is one of the fastest growing industries in the world. The need for enhanced disease resistance, feed efficiency and growth performance of the cultured organism is substantial for various sectors of this industry. If growth performance and feed efficiency are increased in commercial aquaculture, then the costs of production are likely to be reduced. Also if more fish are able to resist disease and survive until they are of marketable size, the subsequent cost of medication and overall production costs would be reduced drastically. It has been documented in a number of food animals that their gastrointestinal microbiota plays important roles in affecting the nutrition and health of the host organism. Thus, various means of altering the intestinal microbiota to achieve favorable effects such as enhancing growth, digestion, immunity and disease resistance of the host organism have been investigated in various terrestrial livestock as well as in humans. Dietary supplementation of prebiotics, which are classified as non-

This dissertation follows the style of the Journal of the World Aquaculture Society.

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digestible food ingredients that beneficially affect the host by stimulating growth and/or activity of a limited number of health-promoting bacteria such as *Lactobacillus* and *Bifidobacter* spp. in the intestine, while limiting potentially pathogenic bacteria such as *Salmonella*, *Listeria* and *Escherichia coli*, have been reported to favorably affect various terrestrial species; however, such information is extremely limited to date for aquatic organisms. Effects of probiotics, defined as live microbial feed supplements, on gastrointestinal microbiota have been studied in some fishes, but the primary application of microbial manipulations in aquaculture has been to alter the composition of the aquatic medium. In general, the gastrointestinal microbiota of fishes including those produced in aquaculture has been poorly characterized, especially the anaerobic microbiota. Therefore, more detailed studies of the microbial community of cultured fish are needed to potentially enhance the effectiveness of prebiotic and probiotic supplementation. This review summarizes and evaluates current knowledge of intestinal microbial ecology of fishes, the various functions of this intestinal microbial community and the potential for further application of prebiotics and probiotics in aquaculture.

Introduction

Over the past decade, production of seafood via aquaculture has continued to exhibit sustained expansion throughout the world, and in 2002 provided approximately 30% of all seafood products (Anon. 2005). Further expansion of commercial aquaculture is anticipated to provide an increasing percentage of the seafood demanded by the world's growing population as most capture fisheries of the world are already at or

beyond maximum sustainable levels (FAO Fisheries Department 2002). Continued expansion of aquaculture will be enhanced by increases in production efficiency and intensity, where considerable opportunity exists for further advancements. Intensification of production has revealed a multiplicity of constraints and accentuated our limited understanding of many basic biological and ecological processes. Production intensification increases the dependence on prepared diets to provide all the required nutrients to support optimal growth and health of the cultured organisms. As such, prepared diets constitute the largest variable cost in intensive fish production, typically ranging from 40 to 60% (Webster and Lim 2002). Prepared diets not only provide essential nutrients to support growth and development of the cultured organism, but they also may influence compositional attributes of the organism as well as nutrient excretion into the environment. One of the most promising paradigms recently to emerge from research with prepared diets concerns their use as functional feeds, in which their influence is extended to the cultured organism's health and resistance to stress and disease-causing agents (Gatlin 2002).

Dietary supplements such as probiotics, originally defined as live microbial feed supplements that beneficially affect intestinal microbial balance of the host organism (Fuller 1989), have received heightened attention in aquaculture over the past several years (Gatesoupe 1999; Gatlin 2002; Irianto and Austin 2002). More recently, prebiotics which are classified as non-digestible food ingredients that beneficially affect the host by stimulating growth and/or activity of a limited number of beneficial bacteria in the gastrointestinal (GI) tract such as *Lactobacillus* and *Bifidobacter* spp., while limiting

potentially pathogenic bacteria such as *Salmonella*, *Listeria* and *Escherichia coli*, have become the focus of intensified research in terrestrial animals (Manning and Gibson 2004). The microbiota that is affected by prebiotics plays integral roles in numerous processes including growth, digestion, immunity and disease resistance of the host organism as demonstrated in poultry (Patterson and Burkholder 2003), other terrestrial livestock and companion animals (Flickinger et al. 2003), as well as in humans (Gibson and Roberfroid 1995). At this time, the application of prebiotics in aquaculture has been rather limited but holds considerable potential. However, to effectively apply prebiotics or probiotics in aquaculture, the microbial community of finfishes has to be better characterized and understood.

Gastrointestinal Microbiota of Fishes

The GI tract of invertebrates and vertebrates provide habitat for a diverse ecosystem of microorganisms. These microorganisms play an important role in the health and nutrition of the host. The vertebrate GI tract is predominantly an anaerobic environment, and in fishes the GI tract's microbial community of fishes, especially the anaerobic microbial community, is poorly studied and understood. The majority of studies characterizing the fishes' intestinal microbial community have been aerobic studies (reviewed in Cahill 1990; Ringø 1993; Spanggaard et al. 2000; and Huber et al. 2004), which consequently determines the dominant facultative anaerobic bacteria, but does not cultivate the strictly anaerobic bacteria. Using aerobic methods to culture bacteria have led some investigators to conclude that anaerobic bacteria play a minor

role in the GI tract's microbial community of fishes. For example, Spanggaard et al. (2000) concluded that the anaerobic microbial community was a minor component of the GI tract's microbial community of rainbow trout *Oncorhynchus mykiss* because the plate count estimates and direct count estimates produced similar results. However, it is impossible to determine microbial species using microscopic direct count methods and even if the estimates were similar, the species counted and cultured could be different.

There have been few attempts to isolate and characterize obligate anaerobes from the GI tract of fishes; the first was reported by Trust et al. (1979). They isolated bacteria from the GI tract of grass carp *Ctenopharyngodon idella*, goldfish *Carassius auratus* and rainbow trout. The bacteria identified were largely unknown and the community structure, fermentation capabilities and interactions with the host were not examined. Anaerobic bacteria were next isolated from the intestinal tract of various freshwater fish species (Sakata et al. 1980). This study only compared the number of anaerobic and aerobic bacteria present in the GI tract of these species as none of the isolated bacteria were identified. However in that study, anaerobic jars were used to isolate the anaerobes, but this method does not isolate strict anaerobes as the atmosphere inside the jar would start out aerobic and take several hours to become completely anaerobic (Riley et al. 1999). The intestinal microbiota of farm-raised channel catfish *Ictalurus punctatus* also has been examined (VanVuren 1998). The dominant bacterial species isolated was not specifically described, but was cellulolytic and thus may help the host to digest plant materials. Ramirez and Dixon (2003) isolated anaerobes from the GI tract of oscars *Astronotus ocellatus*, angelfish *Pterophyllum scalare* and southern flounder *Paralichthys*

lethostigma and found that most of the bacterial species could be classified as *Clostridium*, *Bacteriodes*, *Porphyromonas*, and *Fusobacterium*.

Anaerobic studies of the GI tract of fishes are essential to fully characterize the microbial community of the host and evaluate the effects of dietary supplements designed to stimulate specific beneficial bacteria. The limited use of strict anaerobic techniques has lead to some faulty conclusions. For example, Weinstein et al. (1982) found cellulase activity occurring in the intestinal tracts of pinfish *Lagodon rhomboides* but were unable to cultivate any cellulase-producing bacteria from the GI tract and therefore concluded the source of cellulase activity was likely to be the pinfish. However their sampling procedure for intestinal microbiota exposed the intestinal contents to oxygen before transfer to an anaerobic bag and thus any obligate anaerobes might have been killed. In addition, the samples were placed on dry ice (-20°C) for up to 96 h, which is lethal to some microorganisms (Atlas and Bartha 1993). Weinstein et al. (1982) observed that the intestinal tissue appeared to be the source of the cellulase and the intestinal contents had very little if any cellulase activity. However, even after washing the GI tissues thoroughly with water, cellulolytic bacteria may still adhere to the walls of the intestinal lumen and exhibit cellulase activity. Luczkovich and Stellwag (1993) and Stellwag et al. (1995) subsequently discovered that pinfish have cellulolytic bacteria in their intestinal tract that are obligate anaerobes.

Role of Anaerobic Gastrointestinal Microflora

Enzyme activity of anaerobic bacteria isolated for the GI tract of fishes has been examined to a limited extent. Amylase activity was examined in five species of fishes: Ayu *Plecoglossus altivelis*, common carp *Cyprinus carpio*, channel catfish, Japanese eel *Anguilla japonica* and tilapia *Oreochromis niloticus* (Sugita et al. 1997). The activity of amylase was found to occur in a higher percentage of the isolated anaerobic bacteria (68.4%) as compared to the isolated aerobes (20%). Thus the anaerobic microbiota of the GI tract may play an important function in the digestive capabilities of the host. The enzymatic activity of anaerobic bacteria isolated from Oscars, angelfish, and southern flounder varied greatly, with some enzyme activity present in the microbial community that was not endogenous to the host (Ramirez and Dixon 2003); however, specific microbial processes such as fermentation, pathogen inhibition or effects on the digestive capability of the host species were not examined (Ramirez and Dixon 2003). Anaerobic carboxymethylcellulase-producing bacteria have been isolated from the intestinal tract of pinfish (Luzckovich and Stellwag 1993) and free cellulose has been shown to be degraded within the intestinal tract of pinfish (Burr 1999). Thus recalcitrant molecules (such as fiber) could become an energy source for monogastric species with enzymatic assistance from endogenous microbiota. However, the extent to which the microbial enzymes assist the fish in obtaining energy from otherwise indigestible components of the diet has not been quantified.

Manipulation of Fish GI Microbiota to Benefit the Host

In recent years, considerable benefits have been established in terrestrial animals by feeding potentially beneficial bacteria as probiotics or adding supplements to the diet to alter the intestinal environment and favor the establishment of certain microorganisms. The use of probiotics have been studied most extensively in terrestrial vertebrates such as pigs (Sakata et al. 2003; Gardiner et al. 2004), chickens (Nisbet, 2002; Patterson and Burkholder 2003) and humans (Fioramonti et al. 2003), but to a more limited extent in fishes (reviewed by Gatesoupe 1999; Verschuere et al. 2000; Irianto and Austin, 2002). The GI tract microbial community of the host organism fed a probiotic becomes readily dominated by the probiont; however, the probiont typically disappears within days after withdrawal of the probiotic as demonstrated in chickens (Netherwood et al. 1999).

Probiotics have been shown to have numerous favorable effects on the host including increased nutrient digestion. For example, probiotics have been used to aid in the digestion of lactose by people without lactase (Jiang and Savaiano 1997). In juvenile turbot *Scophthalmus maximus*, growth was significantly increased with the addition of *Lactobacillus* spp. to the diet (Gatesoupe 1991). Nitrogen retention of turbot also was reported to increase when the diet was supplemented with *Vibrio proteolyticus* (De Schrijver and Ollevier 2000).

Probiotics also have been reported to inhibit diseases of the GI tract (Mao et al. 1996; Ichikawa et al. 1999) and aid in the development of the GI tract immune system (Fukushima et al. 1999; Rodrigues et al. 2000). Probiotics also may provide benefits for the GI tract itself by impeding degradation of the intestinal mucus (Rojas and Conway

1996; Zhou et al. 2001). In livestock production, probiotics mainly have been used to enhance the disease resistance of the host to bacterial pathogens by modifying the microbial community of the GI tract (Petterson and Burkholder 2003). Pathogenic microorganisms infect terrestrial animals through the GI tract and competitive exclusion cultures have been reported to inhibit diseases in both swine and poultry (Nisbet 2002), including inhibition of *Campylobacter jejuni* colonization in chicks (Schoeni and Wong 1994).

Lactic acid bacteria have been the most commonly used probiont in humans (reviewed in Fioramonti et al. 2003), poultry (reviewed in Patterson and Burkholder 2003), and swine (Ohashi et al. 2004). Lactic acid bacteria also have received considerable attention as probiotics in fishes (Ringø and Gatesoupe 1998; Gildberg and Mikkelsen 1998; Hagi et al. 2004) (Table 1). For example, lactic acid bacteria included in the diet of Atlantic cod *Gadus morhua* was found to increase the survival of the host when challenged with the bacterial pathogen *Vibrio anguillarum* (Gildberg and Mikkelsen 1998). Production of acetate and lactate by lactic acid bacteria has been shown to inhibit the growth of several species of *Vibrio* (Vázquez et al. 2005). Enhanced survival and increased specific and non-specific immune responses have been demonstrated in rainbow trout (Nikoskelainen et al. 2003; Panigrahi et al. 2005) and

Table 1. Summary of finfish responses to dietary supplementation with various probiotics.

Designations of (o), (+) and (-) indicate neutral, positive and negative responses, respectively, associated with the various measurements.

| Probiotic | Species | Dose and timing | Measured response | Reference |
|--|-------------------|---|---|--|
| Live bacteriophage | Ayu | 10^7 CFU/g diet once | Resistance to <i>Pseudomonas plecoglossicida</i> + | Park et al. (2000) |
| <i>Aeromonas media</i> strain A199 | Eel | 10^5 CFU/ml water added daily | Resistance to <i>Saprolegnia parasitica</i> + | Lategen et al. (2004) |
| <i>Bacillus subtilis</i> and <i>B. licheniformis</i> | Rainbow trout | 4×10^4 spore/g diet for 42 d | Resistance to <i>Yersinia ruckeri</i> + | Raida et al. (2003) |
| <i>Bacillus subtilis</i> and <i>Lactobacillus delbrückii</i> | Gilthead seabream | 0.5×10^7 cfu/g to 1.0×10^7 cfu/g to diet for 21 d | Cellular innate immune response + | Salinas et al. (2005) |
| <i>Carnobacterium divergens</i> | Atlantic cod | for 21 d dose not given | Survival + Resistance to <i>Vibrio anguillarum</i> + | Gildberg et al. (1997) Gildberg et al. (1998) |

Table 1. Continued

| Probiotic | Species | Dose and timing | Measured response | Reference |
|---|-----------------------|---|---|-----------------------------|
| <i>Carnobacterium inhibens</i> | Rainbow trout | 10^6 - 10^8 cell/g diet for 7-14 d | Resistance to <i>Aeromonas salmonicida</i> + Immune response + | Irianto and Austin (2002b) |
| <i>Debaryomyces hansenii</i> | European sea bass | 7×10^5 CFU/g diet | amylase secretion +; survival +; growth - | Tovar et al. (2002) |
| <i>Enterococcus faecium</i> | European eel | 0.1% diet for 14 d | Resistance to <i>Edwardsiella tarda</i> + | Chang and Liu (2002) |
| <i>Lactobacillus spp.</i> , <i>Lactococcus ssp.</i> , <i>Lueconostoc ssp.</i> | Pathogens from Turbot | <i>In vitro</i> challenges to pathogenic bacteria | Resistance to <i>Vibrio spp.</i> , <i>V. anguillarum</i> + | Vásquez et al. (2005) |
| <i>Lactobacillus rhamnosus</i> | Rainbow trout | 10^9 cell/g diet for 51 d | Resistance to <i>Aeromonas salmonicida</i> + | Nikoskelainen et al. (2001) |
| <i>Lactobacillus rhamnosus</i> | Rainbow trout | 9×10^4 , 2.1×10^6 , 2.8×10^8 , 9.7×10^{10} CFU/g diet | Immune responses + | Nikoskelainen et al. (2003) |

Table 1. Continued

| Probiotic | Species | Dose and timing | Measured response | Reference |
|-------------------------|--------------|--|------------------------------|----------------|
| <i>Lactobacillus</i> | Rainbow | 10 ¹¹ CFU/g diet for | Immune response + for | Panigrahi et |
| <i>rhamnosus</i> (heat- | trout | 30 days | live and freeze dried | al. (2005) |
| killed, live and | | | Immune response o for | |
| freeze dried) | | | heat killed | |
| <i>Pseudomonas</i> | Salmon | 1×10 ⁵ to 1×10 ⁶ | Resistance to | Gram et al. |
| <i>fluorescens</i> | <i>Salmo</i> | CFU/ml water | <i>Aeromonas salmonicida</i> | (2001) |
| Strain AH2 | <i>salar</i> | | o | |
| <i>Saccharomyces</i> | Nile | 0.1% diet for 63 d | Weight gain and feed | Lara-Flores et |
| <i>cerevisiae</i> | tilapia | | efficiency + | al. (2002) |
| <i>Vibrio fluvialis</i> | Rainbow | 10 ⁶ -10 ⁸ cell/g diet | Resistance to | Irianto and |
| | trout | for 7-14 d | <i>Aeromonas salmonicida</i> | Austin |
| | | | + | (2002b) |
| | | | Immune response + | |

Adapted from Gatlin, D. M., III and P. Li. 2005. Use of diet additives to improve nutritional value of alternative protein sources. In: C. Lim and C. Webster (eds.)

Alternative Protein Feedstuffs for Aquaculture. Haworth Press.

gilthead seabream (Salinas et al. 2005) fed lactic acid bacteria. Although lactic acid bacteria have been most widely studied probiotic, *Aeromonas media* has been reported to decrease saprolegniosis in challenged eels *Anguilla australis* (Lategen et al. 2004).

While probiotics have been successfully shown to decrease mortality in larval and pathogen-challenged fishes, as well as provide additional enzymes to potentially aid the host in digestion, the use of probiotics is potentially limited for several reasons. In particular, the viability of these probiotic microbes may be affected by the harsh conditions of extrusion or pellet manufacturing. There also may be possible regulatory issues to limit microbial supplements in the diet. Thus, prebiotic supplements have received heightened attention potentially offering the same benefits of probiotics without the addition of live bacteria to the diet.

Prebiotics

Prebiotics have been defined as non-digestible diet components that are metabolized by specific microorganisms beneficial to the health and growth of the host (Gibson and Roberfroid 1995; Manning and Gibson 2004). Some of the more common prebiotics established to date include fructooligosaccharide (FOS), transgalactooligosaccharide (TOS) and inulin (Vulevic et al. 2004). Prebiotics shift the microbial community to one dominated by beneficial bacteria, such as *Lactobacillus* spp. and *Bifidobacterium* spp. (Biecklecka et al. 2002; Patterson and Burkholder 2003; Manning and Gibson 2004). Prebiotics have been used in humans (reviewed by Gibson and Roberfroid 1995; Manning and Gibson 2004; Rastall 2004), poultry (Patterson and

Burkholder 2003) and pigs (Smiricky-Tjardes et al. 2003; Konstantinov et al. 2004).

These studies have reported that prebiotics can modify the GI tract microbial community to enhance non-specific immune responses (Bailey et al. 1991), increase fermentation products (Smiricky-Tjardes et al. 2003), as well as improve mineral uptake (Bongers and van der Huevel 2003) and livestock performance indices such as protein efficiency ratio and feed conversion ratio (Kirkpinar et al. 2004).

Prebiotics may alter the fermentation products of the GI tract as demonstrated by Smiricky-Tjardes et al. (2003) who reported that TOS increased the concentrations of the volatile fatty acids (VFAs) propionate and butyrate in the small intestines of swine. However, in that study nutrient digestibility was reported to be lower even though the intestinal microbiota had shifted to a more beneficial community for the host (Smiricky-Tjardes et al. 2003). Pigs fed the prebiotic gluconic acid showed an increase in butyrate production compared to pigs fed a glucose-containing diet (Tsukahara et al. 2002). Increasing concentrations of VFAs in the GI tract also have been shown to inhibit pathogen colonization (Manning and Gibson 2004; Vázquez et al. 2005). However, other studies have reported that prebiotics do not have any effect on pattern of VFA production or the concentration detected in the GI tract (Cummings et al. 2001; Flickinger et al. 2003).

Increased bioavailability of glucose and trace elements with the inclusion of prebiotics in the diet has been reported (Breves et al. 2001; Bongers and van den Heuvel 2003). Glucose uptake was significantly higher in the GI tract of pigs fed diets supplemented with prebiotics (Breves et al. 2001). The increased availability of trace

elements was attributed to decreasing the pH of the intestinal tract due to the increased concentrations of VFAs (Bongers and van den Heuvel 2003). There also may be an osmotic effecting which the exchange of protons and possible decreases in proteins such as calcium-binding protein may increase the availability of trace elements in the small intestine (Bongers and van den Heuvel 2003).

Prebiotics also have been reported to increase feed efficiency and weight gain in broiler chicks, while mixed results have been reported in pigs (Flickinger et al. 2003). When fed FOS, broilers had higher carcass weight and lower fat deposition when compared to those fed a control diet (Flickinger et al. 2003). A prebiotic-enzyme preparation added to a broiler diet containing poultry by-product meal up to 20% by weight was shown to increase protein efficiency and feed conversion (Kirkpinar et al. 2004).

The common prebiotic FOS was reported to lessen *Salmonella* Typhimurium in the GI tract of chickens when included at 0.75% in the diet (Bailey et al. 1991). The same study also demonstrated that *Salmonella* Typhimurium could not grow when FOS was the sole carbon source. A diet supplemented with FOS also has been reported to lessen infestation of intestinal worms in the GI tract of pigs (Petkevicius et al. 1997).

Despite the potential benefits to health and performance as noted in various terrestrial species, the use of prebiotics with fishes has been poorly studied to date (Table 2). In the earliest of studies with fish, certain nutrients such as linoleic acid, linolenic acid, and soluble carbohydrate were investigated as to their effects on the

Table 2. Potential prebiotics to be evaluated in finfish aquaculture.

| Prebiotic | Bacteria Selected for | References | Bacterial genus detected in fish |
|------------------|----------------------------|---|-------------------------------------|
| IMO ^A | <i>Lactobacillus</i> spp | Chung and Day 2004 | Yes |
| IMO ^A | <i>Bifidobacterium</i> spp | Chung and Day 2004 | No |
| FOS ^B | <i>Lactobacillus</i> spp | Sghir et al. (1998), Kaplan and Hutkins (2000), Swanson et al. (2002a), Steer et al. (2003) | Yes |
| FOS ^B | <i>Bifidobacterium</i> spp | Sghir et al. (1998), Kaplan and Hutkins (2000), Steer et al. (2003) | No |
| MOS ^C | <i>Lactobacillus</i> spp | Swanson et al.(2002b) | Yes |
| MOS ^C | <i>Bifidobacterium</i> spp | Swanson et al.(2002b) | No |

^AIMO = isomaltooligosaccharide^BFOS = fructooligosaccharide^CMOS = mannanoligosaccharide^DGOS = glactooligosaccharide

aerobic/facultative anaerobic intestinal microbiota of Arctic char *Salvelinus alpinus* (Ringø 1993; Ringø et al. 1998; Ringø and Olsen 1999). When linoleic acid was supplemented to the diet of Arctic char the total viable counts increased by an order of magnitude (10 fold) as compared with fish fed a diet without linoleic acid (Ringø 1993). Adding linoleic acid to the diet altered the intestinal microbial community by inhibiting the growth of *Lactobacillus* sp. and enhancing the growth of *Aeromonas* sp., *Pseudomonas* sp., and *Vibrio* sp. Polyunsaturated fatty acids of the n-3 and n-6 series also were shown to alter the microbial population of Arctic char, with the lactic acid bacteria *Carnobacterium* spp. being the dominant facultative anaerobe cultivated (Ringø et al. 1998). The amount of carbohydrates included in the diet of Arctic char was found to affect the diversity of the microbial population, but not the total numbers of bacteria isolated (Ringø and Olsen 1999). In these studies; the anaerobic microbiota, a potentially important constituent of the GI tract, was not examined and neither were specific responses of the host.

The effects of a potential prebiotic (GroBiotic[®]-A) was most recently investigated in hybrid striped bass *Morone chrysops* x *M. saxatilis* (Li and Gatlin 2004; Li and Gatlin 2005). GroBiotic[®]-A is a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products. Li and Gatlin (2004, 2005) found that fish fed a diet containing GroBiotic[®]-A had a significantly higher feed efficiency and significantly lower mortality when challenged with the bacterial pathogens *Streptococcus iniae* and *Mycobacterium marinum*. However, the intestinal microbial community was not investigated to determine if GroBiotic[®]-A altered its composition. Recent *in vitro*

studies in our laboratory have confirmed a shift in the intestinal microbial population of red drum *Sciaenops ocellatus* in the presence of GroBiotic[®]-A based on denaturing gradient gel electrophoresis (unpublished data).

Based on the findings to date with terrestrial animals and to a more limited extent with fish, prebiotics have the potential to enhance numerous biological responses while lowering mortality due to microbial pathogens. However, the anaerobic intestinal tract microbiota of commercially important fishes, such as channel catfish, hybrid striped bass, tilapia, and salmonids need to be investigated to determine if there are any particular bacterial species to be enhanced with the use of prebiotics. Even increasing the production of VFAs in the GI tract would possibly benefit the host by recovering some of the “lost” energy from indigestible dietary constituents and by inhibiting potential pathogenic bacteria (Manning and Gibson 2004; Vázquez et al. 2005). The VFAs produced are also indicative of the microbial population present in the GI tract (Nisbet et al. 1996; Nisbet 2002)

Under aerobic conditions oxygen is the terminal electron acceptor of catabolism, but in an anaerobic environment, the intestinal microbiota needs a terminal electron acceptor other than oxygen. Anaerobic microbes produce VFAs that contain one to seven carbons and are metabolic end products of anaerobic metabolism (Atlas and Bartha 1993). Common VFAs include acetic acid (acetate), propionic acid (propionate), butyric acid (butyrate), formic acid (formate), lactic acid (lactate), butyric acid (butyrate), and valeric acid (valerate).

Herbivorous fishes such as sea chubs, *Kyphosus cornellii* and *K. sydneyanus*, were the first species shown to have VFAs as bacterial metabolic by-products in their intestinal tracts (Rimmer and Weibe 1987, Choat and Clements 1998). Other fishes that have been found with bacterial VFAs in their intestinal tracts include tilapia *Oreochromis mossambicus* (Titus and Ahern 1988), the reef fish *Odax pullus* (Clements et al. 1994), and fishes from kelp forests including the monkeyfaced prickleback, *Cebidichthys violaceus*, the halfmoon, *Medialuna californiensis*, and the sea chubs, *Kyphosus bigibbus* and *K. vaigiensis* (Kandel et al. 1994).

Rimmer and Weibe (1987) found VFAs were restricted to the hind-gut caecum of *Kyphosus sydneyanus* and *K. kyphosus*, but did not report which VFAs were present or their concentrations. Kandel et al. (1994) found that in the cool-temperate half-moon, which feeds predominantly on kelp, the only VFA detected in the intestinal tract was acetate. Smith et al. (1996) looked at VFA concentrations in two temperate freshwater omnivorous fishes (common carp and gizzard shad *Dorosoma cepedianum*) and a carnivorous temperate freshwater fish (largemouth bass *Micropterus salmoides*). The largemouth bass had the highest concentration of VFAs in the intestinal tract of all three species, especially during the summer months, thus demonstrating that both carbohydrates and protein substrates could be fermented. Acetate was detected in largemouth bass in the greatest concentrations (33.5 mmol l^{-1}) and composed over 88 % of all VFAs detected. Acetate was found in the greatest concentrations in the other two species as well, composing 82 % of all VFAs in gizzard shad and 94 % of VFAs in common carp. Propionate had the next highest concentration in all species, comprising

approximately 5% of the VFAs detected in the largemouth bass and gizzard shad and 2% in the common carp.

In order to metabolize VFAs, they must be transported across the intestinal membrane. Titus and Ahern (1988) demonstrated that tilapia *Oreochromis mossambicus*, not only possessed significant VFA concentrations, but also had the ability to actively transport acetate across their intestinal membrane. This active transport mechanism for acetate, which is possibly coupled to the bicarbonate ion (Titus and Ahern 1991), also has some affinity to transport other VFAs across the brush border membrane.

Conclusions

There are several questions that must be answered by more comprehensively evaluating probiotics and/or prebiotics as dietary supplements for fish under aquacultural conditions. There is limited knowledge of the anaerobic microbial community in the GI tract of various fish species which raises the question: Are the lactic acid bacteria beneficial to the fish and are *Bifidobacterium* present in GI tract? The microbial communities in the GI tract of fishes studied to date have been reported to be mainly acetogenic. Can prebiotics increase acetate and lactate concentrations to inhibit pathogen colonization? Do prebiotics increase non-specific or specific immune responses in fishes? Probiotics and prebiotics have not always given consistent results in enhancing performance indices of livestock. This aspect of probiotic and/or prebiotic use also will need to be addressed over a broad range of aquacultural conditions.

In summary, prebiotics have been reported to have numerous beneficial effects in terrestrial animals such as increased disease resistance and improved nutrient availability. If these types of responses are manifested in fishes, then prebiotics have much potential to increase the efficiency and sustainability of aquacultural production. Therefore, comprehensive research to more fully characterize the intestinal microbiota of prominent fish species and their responses to prebiotics is warranted.

CHAPTER II

IN VITRO ASSESSMENT OF GROBIOTIC[®]-A, BREWER'S YEAST AND FRUCTO-OLIGOSACCHARIDE AS PREBIOTICS FOR THE RED DRUM *Sciaenops ocellatus*

Synopsis

The current study examined the effects of brewers yeast, fructo-oligosaccharide (FOS), and GroBiotic[®]-A, a mixture of partially autolyzed brewers yeast, dairy components and dried fermentation products, on the intestinal microbial community of red drum, *Sciaenops ocellatus*. Gastrointestinal (GI) tracts were aseptically removed from three sub-adult red drum previously maintained on a commercial diet, and the excised tracts then were placed in an anaerobic chamber. Intestinal contents were removed, diluted and incubated *in vitro* in one of four liquid media: normal diet alone, diet + 2% (w/w) GroBiotic[®]-A, diet + 2% brewers yeast, and diet + 2% FOS. After 24 and 48 h of incubation at 25°C, supernatants were removed for short chain fatty acid (VFA) analysis and DNA was extracted for denaturing gradient gel electrophoresis (DGGE) analysis. Polymerase chain reaction (PCR) was performed on a highly conserved region of MC (microbial community) 16S rDNA and the amplicons were subjected to DGGE. The MC fingerprint was used to distinguish microbial populations. The intestinal contents incubated with GroBiotic[®]-A had significantly ($p < 0.05$) higher acetate and total VFA concentrations at 48 h compared to the other treatments. DGGE analysis demonstrated that the microbial community was significantly altered by Grobiotic[®]-A and brewers

yeast. Prebiotic application appears to have considerable potential as a means to enhance the efficiency, safety and profitability of aquaculture in the United States.

Introduction

Recently there has been increased interest in altering the intestinal microbiota of animals by introducing beneficial bacteria to the gastrointestinal (GI) tract or adding supplements to the diet. There are two general approaches used to modify the GI tract bacteria. The first approach is the use of probiotics, which are viable microorganisms that benefit the host (Fioramonti et al., 2003). Probiotics have been studied in pigs (Sakata et al. 2003; Gardiner et al. 2004), chickens (Patterson and Burkholder 2003) and humans (Fioramonti et al. 2003) as well as fishes (reviewed by Gatesoupe 1999, Werschuere et al. 2000, Irianto and Austin 2002, Vine et al. 2004). The second commonly used approach to modify the GI tract microbial community is the addition of prebiotics to the diet. Prebiotics are defined as “nondigestable food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon” (Gibson and Roberfroid 2004). Prebiotics have been found to have beneficial effects in humans (reviewed by Manning and Gibson 1995; Rastall 2004), poultry (Patterson and Burkholder 2003; Chung and Day 2004), and swine (Smiricky-Tjardes et al. 2003; Konstantinov et al. 2004). Reports from these studies reveal that prebiotics can modify the GI tract microbial community to enhance non-specific immune responses (Bailey et al. 1991), increase fermentation products (Smiricky-Tjardes et al. 2003), as well as improve mineral uptake (Bonger and van den

Heuvel 2003), and livestock performance indices such as protein efficiency ratio and feed conversion ratio (Kirkpinar et al. 2004). Smiricky-Tjardes et al. (2003) demonstrated that dietary transglacto-oligosaccharide increased the concentrations of the volatile fatty acids (VFAs) propionate and butyrate in the small intestine of swine. Prebiotics such as oligofructose have been reported to increase bioavailability of glucose and trace elements in the diet Breves et al. 2001; (Bonger and van den Heuvel 2003). Oligofructose has been shown to increase feed efficiency and weight gain in broiler chicks, while mixed results have been seen in pigs (Flickinger et al. 2003). These potential benefits of prebiotics have not been investigated in fishes.

The GI microbial community, especially the anaerobic microbiota, of fishes has been poorly studied and therefore is not well understood. The majority of studies characterizing the microbial community of fish have been aerobic studies (reviewed in Cahill 1990; Ringø 1993; Spanggaard et al. 2000; Huber et al. 2004), which can be useful for determining the dominant facultative anaerobic bacteria, but are not appropriate for assessing the contribution of strict anaerobic bacteria. Using aerobic methods to culture bacteria has led some investigators to conclude that anaerobic bacteria in fish play a minor role in the GI tract microbial community (Spanggaard et al. 2000). Anaerobic studies of the GI tract of fishes are essential to evaluate the effects of the entire microbial community on the host (Burr et al. 2005).

Prebiotics have received considerable attention from the terrestrial livestock industry as a way to improve the disease resistance and to increase growth performance of the host organism. However, little attention has been given to prebiotics in

aquaculture. In fishes, the limited work done with prebiotics has focused on *in vivo* studies. Linoleic acid and other dietary fatty acids, as well as dietary carbohydrate components, have been shown, to alter the aerobic/facultative intestinal microbiota of Arctic charr (*Salvelinus alpinus*), using classical microbiological techniques (Ringø 1993; Ringø et al. 1998; Ringø and Olsen 1999). When linoleic acid was supplemented in the diet of Arctic charr, the total viable counts from the GI tract aerobic/facultative microbial community were increased 10-fold as compared with fish fed a diet without linoleic acid (Ringø et al. 1998).

The purpose of the current study was to determine if Grobiotic[®]-A, brewers yeast and the known terrestrial prebiotic fructo-oligosaccharide (FOS) are effective prebiotics for red drum *Sciaenops ocellatus*. The supplements were evaluated *in vitro* with GI tract inoculum from red drum and the anaerobic microbial community was assessed using denaturing gradient gel electrophoresis (DGGE) and volatile fatty acid (VFA) production.

Methods and Materials

Preparation of samples

The GI tracts of three sub-adult red drum from a recirculating system were aseptically harvested 4 h after the fish were fed a commercial diet containing 40% protein and 10% lipid. The GI tracts were placed into 50-ml conical tubes and transported to an anaerobic chamber (Coy Laboratory Products, Detroit, MI) with an atmosphere of 10% CO₂, 5% H₂, and 85% N₂ gas. The intestinal contents were removed by squeezing and diluted 1:3000 with anaerobic dilution solution (ADS) (K₂HPO₄, 0.45 g/L; KH₂PO₄, 0.45

g/L; $(\text{NH}_4)_2\text{SO}_4$, 0.45 g/L; NaCl, 0.90 g/L; $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, 0.225 g/L; $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$, 0.12 g/L; cysteine, 0.6 g/L; resazurin, 0.02 g/L; and sodium bicarbonate, 1.59 g/L) (Bryant and Robinson 1961; Shermer et al. 1998). The ADS had been placed into the anaerobic hood the previous day to remove any oxygen. Five mls of the diluted intestinal contents or sterile ADS were added to 15-ml tubes containing 0.3 g of diet. This created non-inoculated tubes (without the GI tract microbiota) and inoculated tubes (with the GI tract microbiota). The same commercial diet that was fed to the fish was used as the medium to which the three prebiotics were added to a concentration of 2% on a dry-weight basis. The prebiotics evaluated included GroBiotic[®]-A, a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products (Li and Gatlin 2004) (International Ingredient Corporation, St Louis, MO); partially-autolyzed brewers yeast (International Ingredient Corporation, St Louis, MO); and FOS (Encore Technologies, Plymouth, MN). Each treatment was evaluated in triplicate. The tubes were allowed to incubate at 25°C for 0, 24 and 48 hours. A portion (1 ml) was removed for DNA isolation and PCR at each time interval. The remaining portions of the cultures were centrifuged at 20,000 x g, and 1 ml of the supernatant was used for VFA analysis.

Short chain fatty acid analysis

Volatile fatty acid analysis was done according to the methods of Hinton et al. (1990), as follows: 1 ml of culture was centrifuged at 20,000 x g for 10 min and supernatants were stored at -20°C until analysis was preformed by gas chromatography

using a Shimadzu Gas Chromatograph GC-14A (Shimadzu, Columbia, MD) equipped with a flame ionization detector, an 80/120 CarobpackTM B-DA/ 4% Carbowax[®] 20M (2 m x 2 mm ID) glass column, with an oven temperature of 175°C and detector temperature of 175°C. The flow rate was 24 ml/min. The peak profiles were obtained with a CR501 autointegrator. All samples had 20 mM 2-methylbutyric acid added as an internal standard. The concentrations of the VFA at each incubation interval were subjected to analysis of variance and Duncan's multiple range test for comparison using the Statistical Analysis System (SAS 1985).

DNA isolation and PCR

Genomic DNA was isolated from the initial intestinal content sample and from 1 ml of each subsequent culture with a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) using the method supplied by the manufacturer. The bacteria in each sample were pelleted by centrifuging at 5,000 x g for 10 min. Each pellet was suspended in 180 µl of enzyme solution (20 mg/ml lysozyme, 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1.2% Triton[®]) for 30 min at 37°C.

PCR was conducted using the method of Hume et al. (2003). The use of bacteria-specific PCR primers to conserved regions flanking the variable V3 region of 16S rDNA was used. The primers (50 pmol of each primer; primer 2, 5'-ATTACC GCGGCTGCTGG-3'; primer 3 with a 40 base pair GC clamp (33) 5'-CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGG CAGCAG-3') were mixed with Jump Start Red-Taq Ready Mix (Sigma Chemical

Company, St. Louis, MO) according to the manufacturer's instructions, 250 ng of pooled (83 ng/replicate) template DNA from each of the three replicates was added along with 10 µg of bovine serum albumin (BSA) to help stabilize the reaction. The PCR amplifications were performed on a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). A touchdown PCR program was used to minimize artificial by-products. The program used was as follows: 1) denaturation at 94.9°C for 2 min; 2) denaturation at 94.0°C for 1 min; 3) annealing at 67°C for 45s, -0.5°C per cycle; (to minimize formation of artificial products) (Hume et al. 2003); 4) extension at 72°C for 2 min; repeat steps 2 to 4 for 17 cycles; 6) denaturation at 94°C for 1 min; 7) annealing at 58°C for 45 sec; 8) repeat steps 6 to 7 for 12 cycles; 9) extension at 72°C for 30 min; 10) 4°C final.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was run following the method of Hume et al. (2003) as modified from Muyzer et al. (1993). The amplicons were separated on 8% polyacrylamide gels [(vol/vol) acrylamide-bisacrylamide ratio of 37.5:1 (Bio-Rad, Richmond, CA)] with a 30% to 60% urea-formamide gradient (100% denaturing 7M urea and 40% formamide) using a Dcode System (Bio-Rad, Hercules, CA). The amplicons were mixed with an equal volume of 2X loading buffer [0.05% (wt/vol) bromophenol blue; 0.05% (wt/vol) xylene cyanol; and 70% (vol/vol) glycerol] and 7 µl was loaded into each sample well (16-well comb). The gels were run for 17 hours at 60 volts in 0.5X TAE (20 mM Tris (pH 7.4); 10 mM sodium acetate; 0.5 M EDTA; Bio-Rad, Hercules, CA) at 59°C. Gels were stained for 30 min with SYBR

Green I (USA Amersham Life Sciences, Cleveland, OH) diluted 1:10,000. The fragment analysis pattern relatedness was determined with Molecular Analysis Fingerprinting software (v 1.6; Bio-Rad, Hercules, CA). This analysis is based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages (UPGMA) for clustering. Comparisons between sample band patterns were expressed as a percentage similarity coefficient (%SC).

Sequencing

Six bands from the common bands in the non-inoculated, GroBiotic[®]-A and the brewer's yeast treatments were targeted for sequencing. Plugs from these six bands were removed using sterile 200 µl tips. The plugs were then incubated overnight in Jump Start Red-Taq Ready Mix (Sigma Chemical Company, St. Louis, MO) according to the manufacturer's instructions. The samples were then amplified with the same primers as before except primer 3 did not have the 40 base pair GC clamp. The samples were then reamplified using a blunt end polymerase. The blunt end products were then used in Zero Blunt[®] TOPO[®] PCR Cloning Kit for Sequencing (Invitrogen, Carlsbad, California) according to the methods provided in the kit. Three clones were sequenced and then analyzed using nBLAST at the National Center for Biotechnology Information (NCBI) to identify the genus and/or species.

Results

Short chain fatty acids

The microbial community cultured from the GI tract of red drum was acetogenic (Table 3). After 24-h incubation in an anaerobic environment, acetate production was significantly ($P \leq 0.05$) increased with the addition of GroBiotic[®]-A compared to diet alone or the addition of FOS in the inoculated samples and all of the non-inoculated samples. After 48 h the differences were not significant ($P \geq 0.05$). Propionate production did not vary at any incubation time (Table 3). Butyrate production was significantly ($P \leq 0.05$) increased after 24 h for the sample containing GroBiotic[®]-A compared to the other samples (Table 4). However, after 48 h no differences were apparent. Total volatile fatty production was increased ($P \leq 0.05$) after 24 h for the samples containing GroBiotic[®]-A compared to the other samples (Table 4); differences were not significant after 48 h although samples containing GroBiotic[®]-A had the highest VFA production.

DGGE analysis

The DNA isolated from the 0-h samples was low in concentration and did not amplify and thus was omitted from the DGGE analysis. The cluster analysis separated the samples into two groups. One group was composed of the 24-h samples that differed significantly from the group composed of the 48-h samples (Figure 1; 8%SC). The banding patterns for the 24-h samples indicated that microbial populations were not altered extensively by the addition of the prebiotics. Bacterial populations after 24 h of

Table 3. *In vitro* acetate and propionate production by the intestinal bacteria from red drum cultured under anaerobic conditions at 27°C¹

| | Incubation Time (h) | | | Incubation Time (h) | | |
|--------------------------------|---------------------|----------------------------|--------------|--------------------------|------------|--------------------------|
| | 0 | 24 | 48 | 0 | 24 | 48 |
| | Acetate | | | Propionate | | |
| No inoculum (NI) | 0.77 ± 0.6 | 12.44 ± 11.6 ^B | 33.17 ± 16.5 | 0.83 ± 0.6 ^{AB} | 0.00 ± 0.0 | 0.00 ± 0.0 ^B |
| NI + FOS ² | 0.76 ± 0.4 | 10.31 ± 4.9 ^B | 44.68 ± 26.2 | 0.58 ± 0.4 ^{AB} | 0.00 ± 0.0 | 0.02 ± 0.0 ^B |
| NI + GroBiotic [®] -A | 0.48 ± 0.4 | 10.06 ± 2.9 ^B | 50.41 ± 25.5 | 0.47 ± 0.1 ^B | 0.00 ± 0.0 | 0.42 ± 0.7 ^{AB} |
| NI + Yeast | 0.92 ± 2.2 | 21.45 ± 2.3 ^B | 53.26 ± 15.5 | 2.23 ± 2.2 ^A | 0.00 ± 0.0 | 1.26 ± 1.6 ^A |
| Inoculum (I) | 0.75 ± 0.7 | 16.18 ± 12.9 ^B | 42.58 ± 15.1 | 0.72 ± 0.5 ^{AB} | 0.00 ± 0.0 | 0.00 ± 0.0 ^B |
| I + FOS | 1.19 ± 0.8 | 26.99 ± 12.5 ^B | 51.93 ± 27.9 | 0.77 ± 0.3 ^{AB} | 0.00 ± 0.0 | 0.00 ± 0.0 ^B |
| I + GroBiotic [®] -A | 1.00 ± 0.9 | 88.73 ± 86.9 ^A | 67.85 ± 19.2 | 0.90 ± 0.4 ^{AB} | 0.53 ± 0.9 | 0.00 ± 0.0 ^B |
| I + Yeast | 1.00 ± 0.9 | 42.88 ± 17.0 ^{AB} | 49.51 ± 9.3 | 0.87 ± 0.6 ^{AB} | 0.22 ± 0.4 | 0.16 ± 0.3 ^{AB} |
| Anova P ³ | 0.8527 | 0.1122 | 0.6600 | 0.3700 | 0.5310 | 0.2638 |

¹Within column means ± SD (μmole/ml; n = 3) without a common superscript letter differ significantly (*P*<0.05).

²Fucooligosaccharide

³Means compared within incubation time

Table 4. *In vitro* butyrate and total volatile fatty acid (VFA) production (μmole/ml) by the intestinal bacteria from red drum cultured under anaerobic conditions at 27°C

| | Incubation Time (h) | | | Incubation Time (h) | | |
|--------------------------------|---------------------|-------------------------|--------------|---------------------|----------------------------|--------------|
| | 0 | 24 | 48 | 0 | 24 | 48 |
| | Butyrate | | | Total VFA | | |
| No inoculum (NI) | 0.00 ± 0.0 | 0.00 ± 0.0 ^B | 9.02 ± 8.6 | 1.61 ± 1.2 | 12.44 ± 11.7 ^B | 47.36 ± 23.1 |
| NI + FOS ¹ | 0.00 ± 0.0 | 0.00 ± 0.0 ^B | 6.43 ± 7.1 | 1.34 ± 0.8 | 1.031 ± 4.9 ^B | 55.96 ± 35.5 |
| NI + GroBiotic [®] -A | 0.00 ± 0.0 | 0.00 ± 0.0 ^B | 25.45 ± 35.6 | 0.95 ± 0.4 | 10.06 ± 2.9 ^B | 86.16 ± 54.1 |
| NI + Yeast | 0.04 ± 0.1 | 0.00 ± 0.0 ^B | 15.55 ± 9.3 | 3.19 ± 2.4 | 21.45 ± 2.3 ^B | 76.78 ± 32.7 |
| Inoculum (I) | 0.00 ± 0.0 | 0.00 ± 0.0 ^B | 10.34 ± 17.7 | 1.48 ± 1.3 | 16.18 ± 12.9 ^B | 59.10 ± 32.0 |
| I + FOS | 0.00 ± 0.0 | 0.22 ± 0.4 ^B | 8.37 ± 8.0 | 1.96 ± 0.8 | 27.21 ± 12.8 ^B | 62.34 ± 30.4 |
| I + GroBiotic [®] -A | 0.00 ± 0.0 | 2.73 ± 3.4 ^A | 9.40 ± 14.9 | 1.90 ± 0.8 | 92.47 ± 88.3 ^A | 78.13 ± 16.1 |
| I + Yeast | 0.00 ± 0.0 | 0.28 ± 0.5 ^B | 4.78 ± 2.7 | 1.87 ± 1.19 | 48.80 ± 26.8 ^{AB} | 55.67 ± 12.1 |
| | $P=0.4663^2$ | $P=0.1568^2$ | $P=0.8219^2$ | $P=0.5785^2$ | $P=0.0989^2$ | $P=0.7924^2$ |

¹Within column means ± SD (μmole/ml; n = 3) without a common superscript letter differ significantly ($P<0.05$).

²Fucooligosaccharide

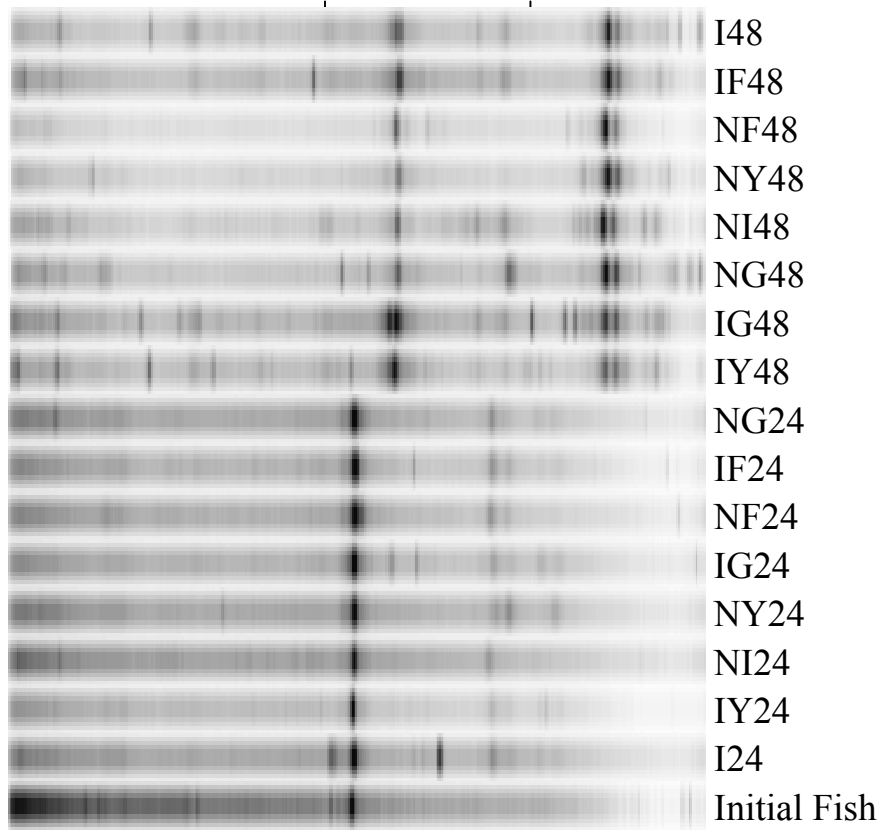
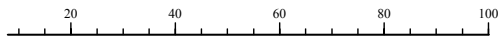
³Means compared within incubation time

anaerobic incubation were either highly related (greater than 90% SC), or could be considered identical (greater than 95% SC). The samples that did not have any diluted digesta added to the tubes (non-inoculated) were similar to the other 24-h samples. The banding pattern of the initial sample, that is, DNA isolated from the original digesta, also was most similar to the 24-h samples (86%SC). The 48-h incubation samples had a greater number of bands than found in the 24-h cultures, possibly indicating a greater proliferation of diverse species. The banding patterns from inoculated samples treated with GroBiotic®-A and brewers yeast were very different from the rest of the 48-h samples with less than 80%SC (Figure 1). Samples with GroBiotic®-A and brewers yeast had close to 80% SC, exhibiting little similarity with each other. The samples with GroBiotic®-A and only sterile ADS also were significantly different from the other samples (80% similarity). This indicated that GroBiotic®-A was possibly stimulating the growth of different bacteria when compared to the other prebiotics. This analysis only examined the culturable anaerobic bacterial population.

Sequencing

The three clones from each band did not all return the same species when run through the Blast database (nr database); however, usually two out of three were the same. The upper common band was most likely *Lactococcus lactis* and the lower common band was *Aeromonas* sp.

Figure 1. Denaturing gradient gel electrophoresis of bacterial 16S rDNA amplicons from red drum intestinal contents. The bar above figure indicates percentage similarity coefficients. NI = non-inoculated + basal diet; NG = non-inoculated + basal diet + 2% GroBiotic[®]-A; NF = non-inoculated + basal diet + 2% FOS; NY = non-inoculated + basal diet + 2% brewers yeast; I = inoculated + basal diet; IG = inoculated + basal diet + 2% GroBiotic[®]-A; IF = inoculated + basal diet + 2% FOS; IY = inoculated + basal diet + 2% brewers yeast. The number indicates incubation time in hours. Initial fish = DNA isolation directly from the intestinal contents.



Discussion

This study is the first to examine the production of VFAs *in vitro* in cultured red drum. Acetate was produced in the highest concentrations and comprised 76-89% of VFA production in inoculated samples and 61-82% in the non-inoculated samples. In the cool-temperate species *Cebidichthys violaceus*, acetate accounted for 100% of the VFAs produced, but in the warm-temperate species *Medialuna californiensis* and two subtropical species, *Kyphosus bibibbus* and *K. vaigeinsis*, acetate production accounted for less than 20% of the VFAs produced (Kandel et al. 1994). *In vitro* acetate production after 48 h was higher than in previous *in vivo* measurements (Smith et al. 1996; Mountfort et al. 2002), indicating that the culturable acetogenic species of microbes are more abundant and have more substrate available than *in vivo* communities. Smith et al. (1996) found that largemouth bass (*Micropterus salmoides*) in summer had acetate concentrations of 33.5 mM in the GI tract. Mountfort et al. (2002) examined temperate herbivorous fishes and found acetate concentrations ranging from 8.3 to 37.5 mM in the GI tracts of three temperate herbivorous species (*K. sydneyanus*, *Odax pullus*, and *Aplodactylus arctidens*). Thus, the concentrations from *in vitro* experiments were approximately 2 to 3 times higher than *in vivo* measurements. Tilapia has been shown to transport VFAs across the intestinal walls (Titus and Ahearn 1988; Titus and Ahearn 1991) and thus the increased VFA production could represent an energy source for the host fish. The increase in VFA production also has been shown to have a beneficial effect on the host immune response by modulating leukocyte activity in both mice and humans (Nilsson et al. 2003).

Based on the variation in VFA production in the individual samples, from this study, it appears that the intestinal community varies from fish to fish. This VFA production variability also could be due to bacterial species not being uniformly distributed among samples or unique members of the intestinal community occurring in individual fish. The VFA profiles did not seem to indicate a change in the microbial community after 48 hours.

In the current study, GroBiotic[®]-A and brewers yeast altered the anaerobic microbial community *in vitro* while the FOS did not, when compared to the microbial community resulting from incubation of the diet alone. The microbial community in the non-inoculated samples containing 2% GroBiotic[®]-A also was altered, indicating that the anaerobic/facultative microbial community already present in the feed can be modified using this prebiotic after 48 hours incubation in anaerobic conditions. The changes to the non-inoculated samples support that GroBiotic[®]-A is a prebiotic because this community is also being modified *in vitro*. GroBiotic[®]-A is an autolyzed yeast product that contains a high level of lactose that can be fermented, thus facilitating the change in the anaerobic community. The lack of detectable change in the FOS samples could be due to the lack of culturable microbes in the red drum GI tract that are adapted to use β -linked carbohydrates, or it is possible that changes in the microbiota were below the detection level of DGGE. The present experiment only examined the culturable community associated with the GI tract of tank-reared red drum. It has been estimated that only 5 to 20% of the species in the GI tract of mammals can be cultured using current media and methods (Moore and Holdeman 1974; Ward et al. 1990).

Results from the current study demonstrated that a single species of bacteria dominated the microbial community after being cultured for 24 h in an anaerobic environment. The two major bands present in all 48-h samples (Figure 1) are most likely *Lactococcus lactis* for the upper band and *Aeromonas* sp. for the lower band. However, it is possible that other species having DNA that is chemically equivalent may have been present. The GI tract samples incubated with brewers yeast and GroBiotic®-A showed a higher complexity when compared to the other samples, indicating that more species were present in these samples. However, after 48 h, many bands were detected, indicating that community bacteria previously undetectable were multiplying in numbers sufficient to reveal DGGE bands. In the inoculated GroBiotic®-A and brewers yeast samples, there appeared to be more bands above the *Lactococcus lactis* band and more bands in between the *Lactococcus lactis* and the *Aeromonas* sp. bands when compared to the inoculated sample without prebiotics. These bands most likely were less numerous species that were able to use the diet additives as a carbon source and able to compete with the more abundant species, thus increasing the complexity of the DGGE profiles. Larval coho salmon *Onchorhynchus kisutch* were reported to have simple DGGE profiles consisting of only four bands with *Pseudomonas* spp. and *Aeromonas* spp. being the dominant species detected (Romero and Navarrete 2006). The uptake of a potential probiotic organism for haddock *Melanogrammus aeglefinus* larva was confirmed using DGGE (Plante et al. 2007). However, the changes in the microbial community related to diet and age were not analyzed and only certain microbial species were determined from each gel. The banding patterns from the Plante et al. (2007) *in vivo* study and the current

in vitro experiment showed differences with the banding patterns from the current study showing a dominant species while the *in vivo* samples showed a more complex pattern with more than one species being dominant at different life stages. This difference is probably due to numerous species that cannot be cultured, but reside in the GI tract of a living host.

In the current study, *Aeromonas* sp. and *Lactococcus lactis* were found to be the dominant species in culture. Using molecular techniques (DNA sequencing and Restriction Fragment Length Polymorphism), Pond et al. (2006) demonstrated that the dominant bacterium of rainbow trout *Oncorhynchus mykiss* was *Clostridium gasigenes*, an anaerobe. Pond et al. (2006) also reported that the intestinal tract bacteria varied from fish to fish, thus complicating potential comparisons among treatments. Ringø et al. (2006) investigated the effects of inulin, containing fructooligosaccharides, on aerobic bacteria associated with the gastrointestinal tract of Arctic charr using DNA sequences of 16S rDNA from 18 culturable species. They determined that inulin changed the community by decreasing the number of bacteria adhering to the GI tract wall. However, in the present *in vitro* study we did not detect any changes in the microbial community of red drum inoculum incubated with 2% FOS. The lack of detectable change could be due to the intestinal contents being removed and the resulting samples obtained likely did not include bacteria that adhere to the intestinal wall.

The addition of brewers yeast and GroBiotic[®]-A to the GI tract contents of red drum altered the microbial community *in vitro*. These *in vitro* results combined with the previous *in vivo* studies conducted with GroBiotic[®]-A and hybrid striped bass indicate

that the detected change in the microbial community may be beneficial to red drum. Beneficial effects such as increased growth performance and disease resistance were conferred to hybrid striped bass fed diets containing GroBiotic[®]-A (Li and Gatlin 2004; 2005). Future studies need to be conducted to identify the microbial species involved and determine if similar changes occur *in vivo*.

CHAPTER III

IN VITRO EVALUATION OF FOUR PREBIOTICS WITH HYBRID STRIPED BASS

Morone chrysops x *M. saxatilis*

Synopsis

Prebiotics have been gaining in popularity as food supplements because they may enhance the natural microbial community that is already present in the gastrointestinal tract (GI) without the need to introduce a foreign organism whose use could be regulated and/or whose viability could be affected by diet processing. The current study examined the effects of four potential prebiotics, GroBiotic[®]-A (consisting of a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products), mannanooligosaccharide (MOS), galactooligosaccharide (GOS), and fructooligosaccharide (FOS) on the intestinal microbial community of hybrid striped bass. Three sub-adult hybrid striped bass previously maintained on a commercial diet containing 40% crude protein and 10% lipid were anesthetized and placed into an anaerobic chamber. Their GI tracts were aseptically dissected and the intestinal contents removed, diluted and incubated with one of eight treatment mediums: diet alone, diet + 1% (by weight) Grobiotic[®]-A, diet + 1% MOS, diet + 1% FOS, diet + 0.5% Grobiotic[®]-A, diet + 0.5% MOS, diet + 0.5% FOS, and diet + 0.5% GOS. The same treatments also were incubated without intestinal contents for comparison. After 24 and 48 h of incubation at 25°C, supernatant was removed for analysis of volatile fatty acids (VFAs) and DNA was extracted from the predominant bacteria for denaturing gradient gel

electrophoresis (DGGE) analysis of a highly conserved region of 16S rDNA to distinguish microbial populations. Results showed that the intestinal contents incubated with 0.5% GroBiotic[®]-A, MOS, and GOS tended to have lower acetate levels but higher butyrate levels at 48 h compared to the diet alone. However, DGGE analysis failed to detect any treatment-related differences in the microbial community of hybrid striped bass.

Introduction

The gastrointestinal (GI) microbial community, especially the anaerobic microbiota, of fishes has been poorly studied and this is not well understood. Most of the studies characterizing the microbial community of fish have been conducted under aerobic conditions (reviewed in Cahill 1990; Gatesoupe 1999; Ringø, 1993; Spanggaard et al. 2000), which can be useful for determining the dominant facultative anaerobic bacteria, but are not appropriate for isolating strict anaerobic bacteria. Using aerobic methods to culture bacteria has led some investigators to conclude that anaerobic bacteria in fish play a minor role in the GI tract microbial community (Spanggaard et al. 2000). Anaerobic studies of the GI tract microbiota of fishes are essential to evaluate the role of the entire microbial community in the host.

The first of a few attempts to isolate strictly anaerobic bacteria from the GI tract of fishes was reported by Trust et al. (1979) working with grass carp *Ctenopharyngodon idella*, goldfish *Carassius auratus* and rainbow trout *Oncorhynchus mykiss*. The bacteria identified were largely unknown and community structure, fermentation capabilities and

interaction of the microbial community with the host were not examined. Anaerobic bacteria were next isolated from the GI tract of various freshwater fish species (Sakata et al. 1980). This study only compared the number of anaerobic and aerobic bacteria present in the GI tract of these species as none of the bacteria isolated were identified. Sakata et al. (1980) used anaerobic jars to isolate the anaerobes, but this method would not isolate strict anaerobes as the atmosphere inside the jar would start out as aerobic and would take several hours to become completely anaerobic. The intestinal microbiota of farm-raised channel catfish (*Ictalurus punctatus*) also have been examined (VanVuren 1998), but the dominant bacterial species isolated was not previously described. This bacterium was cellulolytic and may help the host to use plant material as an energy source.

A healthy GI tract microbial community benefits the host by making it less susceptible to diseases and by possibly aiding nutrient uptake (Fioramonti et al. 2003; Flickinger et al. 2003; Nilsson et al., 2003). There are two general approaches used to modify the GI tract bacteria. The first approach is the use of probiotics, which are live microbes that benefit the host (Fioramonti et al. 2003). Probiotics have been studied in pigs (Gardiner et al. 2004; Smiricky-Tjardes et al. 2003), chickens (Patterson and Burkholder 2003) and humans ((Fioramonti et al. 2003) as well as some fishes (reviewed by Gatesoupe 1999; Verschuere et al. 2000; Irianto and Austin 2002; Vine et al. 2004). The second commonly used approach to modify the GI tract microbial community is the addition of prebiotics to the diet. Prebiotics are defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or

activity of one or a limited number of bacterial species already resident in the colon” (Gibson and Roberfroid 1995). Prebiotics have been found to have beneficial effects in humans (reviewed by Manning and Gibson; 2004; Rastall 2004), poultry (Chung and Day 2004; Patterson and Burkholder 2003), swine (Konstantinov et al. 2004, Smiricky-Tjardes et al. 2003), and fish (Burr et al. 2005). Reports from these studies reveal that prebiotics can modify the GI tract microbial community to enhance non-specific immune responses (Bailey et al. 1991), increase fermentation products (Van Immerseel et al. 2006), as well as improve mineral uptake (Bongers and van der Heuvel 2003) and livestock performance indices such as protein efficiency ratio and feed conversion ratio (Kirkpinar et al. 2004). Smiricky-Tjardes et al. (2003) demonstrated that transgalacto-oligosaccharide included in the diet increased the concentrations of the volatile fatty acids (VFAs) propionate and butyrate in the small intestines of swine. Prebiotics such as oligofructose also have been reported to increase bioavailability of glucose and trace elements in the diet (Breves et al., 2001; Bongers and van der Heuvel, 2003) as well as increase feed efficiency and weight gain in broiler chicks, while mixed results have been seen in pigs (Gardiner et al. 2004). Fructo-oligosaccharide inclusion in the diet at 0.75% lessened *Salmonella* Typhimurium in the GI tract of chickens (Bailey et al. 1991). However, little attention has been given to prebiotics in aquaculture.

In fishes, the limited work done with prebiotics has focused on *in vivo* studies. Linoleic acid and other dietary fatty acids, as well as dietary carbohydrate components, have been shown, using classical microbiological techniques (Ringø, 1993; Ringø et al. 1998; Ringø and Olsen 1999), to alter the aerobic/facultative intestinal microbiota of

Arctic charr *Salvelinus alpinus*. When linoleic acid was supplemented in the diet of Arctic charr, the total viable counts from the GI tract aerobic/facultative microbial community were increased 10-fold as compared with the fish fed a diet without linoleic acid (Ringø 1993). Adding linoleic acid to the diet altered the intestinal microbial community by inhibiting the growth of *Lactobacillus* sp. and enhancing the growth of *Aeromonas* sp., *Pseudomonas* sp., and *Vibrio* sp. Polyunsaturated fatty acids also have been shown to alter the microbial population, with the lactic acid bacteria *Carnobacterium* sp. being the dominant facultative anaerobe cultivated (Ringø et al. 1998). The amount of carbohydrates included in the diet of Arctic charr was found to affect members of some microbial population, but not the total numbers of bacteria isolated (Ringø and Olsen 1999). In these studies with Arctic charr, the investigators did not examine how the change in microbial community affected the host. In addition, a potentially important component, the anaerobic microbiota was not examined.

The purpose of the current study was to determine if GroBiotic[®]-A, mannanoligosaccharide (MOS), galactooligosaccharide (GOS) and fructooligosaccharide (FOS) are potentially effective prebiotics for hybrid striped bass (*Morone chrysops* x *M. saxatilis*). The supplements were evaluated *in vitro* with GI tract inoculum from hybrid striped bass and the culturable anaerobic microbial community was assessed using denaturing gradient gel electrophoresis (DGGE) and VFA analysis.

Methods and Materials

The GI tracts of three sub-adult hybrid striped bass from a recirculating system were aseptically harvested 4 h after the fish were fed a commercial diet (Rangen, Inc., Buhl, ID). This diet contained 40% crude protein and 10% lipid and was fed to the fish for 2 weeks prior to sample collection. The fish were slaughtered and placed into an anaerobic chamber after which the intestinal contents were removed by squeezing and diluted 1:1000 with anaerobic dilution solution (ADS) (K_2HPO_4 , 0.45 g/L; KH_2PO_4 ,0.45 g/L; $(\text{NH}_4)_2\text{SO}_4$,0.45 g/L; NaCl , 0.90 g/L; $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, 0.225 g/L; $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$, 0.12 g/L; cysteine, 0.6 g/L; resazurin, 0.02g/L; and sodium bicarbonate, 1.59g/L) (Bryant and Robinson 1961; Shermer et al. 1998). The ADS had been placed into the anaerobic hood the previous day to remove any oxygen. Ten milliliters of the diluted intestinal contents or sterile ADS were added to 15-ml tubes containing 0.3 g of diet to which the various concentrations of prebiotics had been incorporated. These preparations created non-inoculated tubes (without the GI tract microbiota) and inoculated tubes (with the GI tract microbiota). A fishmeal-based diet containing 40% protein and 10% lipid was used as the basal diet to which the following four prebiotics were added: GroBiotic[®]-A, a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products (Li and Gatlin 2004) (International Ingredient Corporation, St Louis, MO); MOS (Alltech Corp. Nicholasville, KY); GOS (Friesland Foods, Zwolle, Netherlands) and FOS (Encore Technologies, Plymouth, MN). Each of the prebiotics was added to the diet at either 0.5% or 1% of dry weight. Each

treatment was evaluated in triplicate. The tubes were allowed to incubate at 25°C for 0, 24 and 48 h. A portion (9 ml) was removed for DNA isolation and PCR at each time point. The remaining portion of the cultures was centrifuged at 20,000 x g, and 1 ml of the supernatant was used for VFA analysis.

Short chain fatty acid analysis

Volatile fatty acid analysis was done according to the methods of Hinton et al. (1990) using a Shimadzu Gas Chromatograph GC-14A (Shimadzu, Columbia, MD) equipped with a flame ionization detector, an 80/120 CarbopackTM B-DA/ 4% Carbowax[®] 20M (2 m x 2 mm ID) glass column, with an oven temperature of 175°C and detector temperature of 175°C. The flow rate was 24 ml/min. The peak profiles were obtained with a CR501 autointegrator. All samples had 20 mM 2-methylbutyric acid added as an internal standard. The concentrations in $\mu\text{mole/ml}$ of VFAs at each incubation interval were subjected to analysis of variance and Duncan's multiple range test for comparison using the Statistical Analysis System (SAS 1985).

DNA isolation and PCR

Genomic DNA was isolated from the initial intestinal content sample (0.2 ml of intestinal contents) and from 1 ml of each subsequent culture with a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) using the method supplied by the manufacturer. The bacteria in each sample were pelleted by centrifuging at 5,000 x g for 10 min. Each pellet was

suspended in 180 µl of enzyme solution (20 mg/ml lysozyme, 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1.2% Triton[®]) for 30 min at 37°C.

PCR was conducted using the method of Hume et al. (2003). The use of bacteria-specific PCR primers to conserved regions flanking the variable V3 region of 16S rDNA was used. The primers (50 pmol of each primer; primer 2, 5'-ATTACC GCGGCTGCTGG-3'; primer 3 with a 40 base pair GC clamp (23) 5'-CGCCCGCCGCGCGCGGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG GCAGCAG-3') were mixed with Jump Start Red-Taq Ready Mix (Sigma Chemical Company, St. Louis, MO) according to the manufacturer's instructions, 250 ng of pooled (83 ng/replicate) template DNA from each of the three replicates was added along with 10 µg of bovine serum albumin (BSA) to help stabilize the reaction. The PCR amplifications were performed on a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). A touchdown PCR program was used to minimize artificial by-products. The program used was as follows: 1) denaturation at 94.9°C for 2 min; 2) denaturation at 94.0°C for 1 min; 3) annealing at 67°C for 45s, -0.5°C per cycle; (to minimize formation of artificial products) (16); 4) extension at 72°C for 2 min; repeat steps 2 to 4 for 17 cycles; 5) denaturation at 94°C for 1 min; 6) annealing at 58°C for 45 sec; 7) repeat steps 6 to 7 for 12 cycles; 8) extension at 72°C for 30 min; 9) 4°C final.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was run following the method of Hume et al. (2003) as modified from Muyzer et al. (1993). The amplicons were

separated on 8% polyacrylamide gels ((vol/vol) acrylamide-bisacrylamide ratio of 37.5:1 (Bio-Rad, Richmond, CA)) with a 30% to 60% urea-formamide gradient (100% denaturing 7M urea and 40% formamide) using a Dcode System (Bio-Rad, Hercules, CA). The amplicons were mixed with an equal volume of 2X loading buffer [0.05% (wt/vol) bromophenol blue; 0.05% (wt/vol) xylene cyanol; and 70% (vol/vol) glycerol] and 7 µl was loaded into each sample well (16-well comb). The gels were run for 17 h in 0.5X TAE (20 mM Tris (pH 7.4); 10 mM sodium acetate; 0.5 M EDTA; Bio-Rad, Hercules, CA) at 59°C and 60 volts. Gels were stained for 30 min with SYBR Green I (USA Amersham Life Sciences, Cleveland, OH) diluted 1:10,000. The fragment analysis pattern relatedness was determined with Molecular Analysis Fingerprinting software (v 1.6; Bio-Rad, Hercules, CA). This analysis is based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages (UPGMA) for clustering. Comparisons between sample band patterns were expressed as a percentage similarity coefficient (%SC).

Sequencing

Plugs from the three major shared bands in each of the non-inoculated, inoculated, and GroBiotic[®]-A treatments were removed from the stained gel using sterile 200-µl pipette tips. The plugs were then incubated overnight in Jump Start Red-Taq Ready Mix (Sigma Chemical Company, St. Louis, MO). The samples were then amplified with the same primers as before except primer 3 did not have the 40 base pair GC clamp. The samples were then re-amplified using a blunt end polymerase. The blunt

end products were then used in Zero Blunt[®] TOPO[®] PCR Cloning Kit for Sequencing (Invitrogen, Carlsbad, California) according to the methods provided in the kit. Three clones were then sequenced using an ABI 3100 Genetic Analyzer (Bio-Rad, Hercules, CA). The sequences were then analyzed using nBLAST at the NCBI to identify the genus or species present.

Results

Volatile fatty acids

The microbial community cultured from hybrid striped bass GI tract was acetogenic (Table 5). After incubation for 24 and 48 h in an anaerobic environment, acetate production was significantly ($P < 0.05$) increased with the addition of the GI tract inoculum when compared to the non-inoculated samples. There were no significant ($P \geq 0.05$) differences among the prebiotic treatments in the inoculated samples. Propionate production was low compared to acetate and butyrate (Table 5). The non-inoculated samples had significantly ($P \leq 0.05$) higher propionate production compared to the inoculated samples after 48 h of incubation. Butyrate production was significantly ($P \leq 0.05$) increased after 48 h for the sample containing 0.5% GroBiotic[®]-A, 1% MOS, and 0.5% GOS compared to the inoculated control (Table 6). The non-inoculated samples were similar to the inoculated samples in butyrate production after 24 and 48 h. Total volatile fatty production was increased ($P \leq 0.05$) after 24 and 48 h for the samples containing GI tract inoculum compared to the non-inoculated samples (Table 6). After 24 h, samples containing GroBiotic[®]-A and MOS had significantly ($P \leq 0.05$) lower total

Table 5. *In vitro* acetate and propionate production ($\mu\text{mole/ml}$) by the intestinal bacteria from hybrid striped bass cultured under anaerobic conditions at 27°C

| | Incubation time (h) | | Incubation time (h) | |
|-------------------------------------|-------------------------|--------------------------|---------------------|----------------------|
| | 24 | 48 | 24 | 48 |
| Treatment | Acetate | | Propionate | |
| No Inoculum (NI) | 0.71 ± 1.23^G | 94.71 ± 9.62^{DEF} | nd ⁶ | 5.87 ± 2.17^A |
| NI + 1% Grobiotic [®] -A | 6.80 ± 3.05^{GF} | 70.20 ± 15.47^{GF} | nd | 2.31 ± 0.94^{BC} |
| NI + 0.5% Grobiotic [®] -A | 16.78 ± 4.05^{GF} | 59.89 ± 11.56^G | nd | 1.30 ± 0.34^C |
| NI + 1% FOS | 14.48 ± 4.29^{GF} | 116.87 ± 49.39^{BCD} | nd | 2.40 ± 0.54^{BC} |
| NI + 0.5% FOS | 20.97 ± 9.46^F | 79.91 ± 32.24^{EFG} | nd | 2.31 ± 0.55^{BC} |
| NI + 1% MOS ³ | 21.81 ± 2.81^F | 112.29 ± 12.65^{CDE} | nd | 3.12 ± 0.86^B |
| NI + 0.5% MOS | 23.31 ± 11.24^F | 96.14 ± 31.91^{DEF} | nd | 3.66 ± 1.85^B |
| NI + 0.5% GOS ⁴ | 17.51 ± 4.69^{GF} | 99.57 ± 17.63^{DEF} | nd | 3.31 ± 1.69^B |
| Inoculum (I) | 113.91 ± 0.62^{AB} | 166.05 ± 5.54^A | 0.18 ± 0.31 | 1.36 ± 0.83^C |
| I + 1% GroBiotic [®] -A | 94.01 ± 11.54^{CD} | 150.62 ± 3.39^{AB} | nd | 0.86 ± 0.32^C |
| I + 0.5% GroBiotic [®] -A | 72.72 ± 10.75^E | 144.69 ± 7.46^{ABC} | 0.05 ± 0.09 | 0.64 ± 0.19^C |
| I + 1% FOS | 125.90 ± 18.10^A | 143.60 ± 3.58^{ABC} | 0.21 ± 0.37 | 0.7 ± 0.15^C |
| I + 0.5% FOS | 104.45 ± 4.85^{BC} | 149.93 ± 2.99^{AB} | 0.24 ± 0.42 | 0.61 ± 0.10^C |
| I + 1% MOS | 85.35 ± 10.23^{DE} | 154.63 ± 3.51^A | 0.31 ± 0.44 | 1.13 ± 0.22^C |
| I + 0.5% MOS | 82.25 ± 4.79^{DE} | 145.62 ± 2.27^{ABC} | nd | 0.83 ± 0.05^C |
| I + 0.5% GOS | 112.02 ± 20.72^{AB} | 149.51 ± 3.78^{AB} | 0.02 ± 0.03 | 0.74 ± 0.03^C |
| Probability ^{1,2} | <0.0001 | <0.0001 | 0.5614 | <0.0001 |

Table 5. continued

¹ Within column means \pm SD ($\mu\text{mole/ml}$; $n = 3$) without a common superscript differ significantly ($P \leq 0.05$).

² Means compared within incubation time

³ fructooligosaccharide

⁴ mannanooligosaccharide

⁵ galactooligosaccharide

⁶ not detectable

Table 6. *In vitro* butyrate and total VFA production (μmole/ml) by the intestinal bacteria from hybrid striped bass cultured under anaerobic conditions at 27°C

| | Incubation time (h) | | Incubation time (h)) | |
|-------------------------------------|-----------------------------|-------------------------------|------------------------------|-------------------------------|
| | 24 | 48 | 24 | 48 |
| Treatment | Butyrate | | Total VFA | |
| No Inoculum (NI) | 0.00 ± 0.00 ^D | 54.83 ± 5.21 ^{ABCD} | 0.71 ± 1.23 ^G | 155.41 ± 12.57 ^{CDE} |
| NI + 1% Grobiotic [®] -A | 0.53 ± 0.54 ^D | 37.16 ± 4.75 ^{DE} | 7.33 ± 2.65 ^{GF} | 110.81 ± 21.73 ^{EF} |
| NI + 0.5% Grobiotic [®] -A | 1.44 ± 0.86 ^{CD} | 26.88 ± 3.54 ^{DE} | 18.22 ± 4.91 ^{GF} | 89.14 ± 13.93 ^F |
| NI + 1% FOS | 4.37 ± 0.96 ^{ABC} | 48.2 ± 15.73 ^{ABCDE} | 18.86 ± 4.61 ^{GF} | 170.04 ± 67.67 ^{BCD} |
| NI + 0.5% FOS | 4.58 ± 0.63 ^{ABC} | 43.16 ± 0.64 ^{BCDE} | 25.55 ± 10.06 ^F | 125.96 ± 33.27 ^{DEF} |
| NI + 1% MOS ³ | 5.65 ± 2.67 ^{AB} | 53.35 ± 10.77 ^{ABCD} | 27.45 ± 2.15 ^F | 171.77 ± 19.20 ^{BCD} |
| NI + 0.5% MOS | 4.10 ± 2.62 ^{ABC} | 40.85 ± 6.33 ^{CDE} | 27.41 ± 13.57 ^F | 142.9 ± 38.50 ^{DE} |
| NI + 0.5% GOS ⁴ | 3.03 ± 1.84 ^{ABCD} | 40.68 ± 16.84 ^{CDE} | 20.53 ± 5.60 ^{GF} | 147.17 ± 35.60 ^{DE} |
| Inoculum (I) | 5.99 ± 0.51 ^A | 41.74 ± 33.32 ^{CDE} | 120.08 ± 0.69 ^{AB} | 209.14 ± 28.90 ^{AB} |
| I + 1% GroBiotic [®] -A | 2.49 ± 0.46 ^{BCD} | 55.19 ± 8.87 ^{ABCD} | 96.49 ± 11.98 ^{CD} | 207.08 ± 10.60 ^{AB} |
| I + 0.5% GroBiotic [®] -A | 1.61 ± 1.57 ^{CD} | 68.41 ± 6.27 ^A | 74.38 ± 12.16 ^E | 214.04 ± 8.15 ^{AB} |
| I + 1% FOS | 5.87 ± 2.68 ^{AB} | 57.64 ± 1.12 ^{ABCD} | 131.99 ± 21.14 ^A | 201.94 ± 4.31 ^{ABC} |
| I + 0.5% FOS | 4.57 ± 0.94 ^{ABC} | 59.84 ± 10.64 ^{ABC} | 109.25 ± 5.89 ^{BC} | 210.38 ± 7.67 ^{AB} |
| I + 1% MOS | 2.89 ± 1.76 ^{ABCD} | 65.23 ± 3.45 ^{AB} | 88.55 ± 24.17 ^{DE} | 222.54 ± 8.39 ^A |
| I + 0.5% MOS | 1.71 ± 0.31 ^{CD} | 62.52 ± 2.80 ^{ABC} | 83.95 ± 4.96 ^{DE} | 208.97 ± 5.02 ^{AB} |
| I + 0.5% GOS | 4.84 ± 3.67 ^{ABC} | 68.87 ± 2.18 ^A | 116.87 ± 24.41 ^{AB} | 219.14 ± 5.26 ^{AB} |
| Probability ^{1,2} | 0.0010 | 0.0018 | <0.0001 | <0.0001 |

Table 6. Continued

¹Within column means \pm SD ($\mu\text{mole/ml}$; $n = 3$) without a common superscript differ significantly ($P \leq 0.05$).

² means compared within incubation time

³ fructooligosaccharide

⁴ mannanoligosaccharide

⁵ galactooligosaccharide

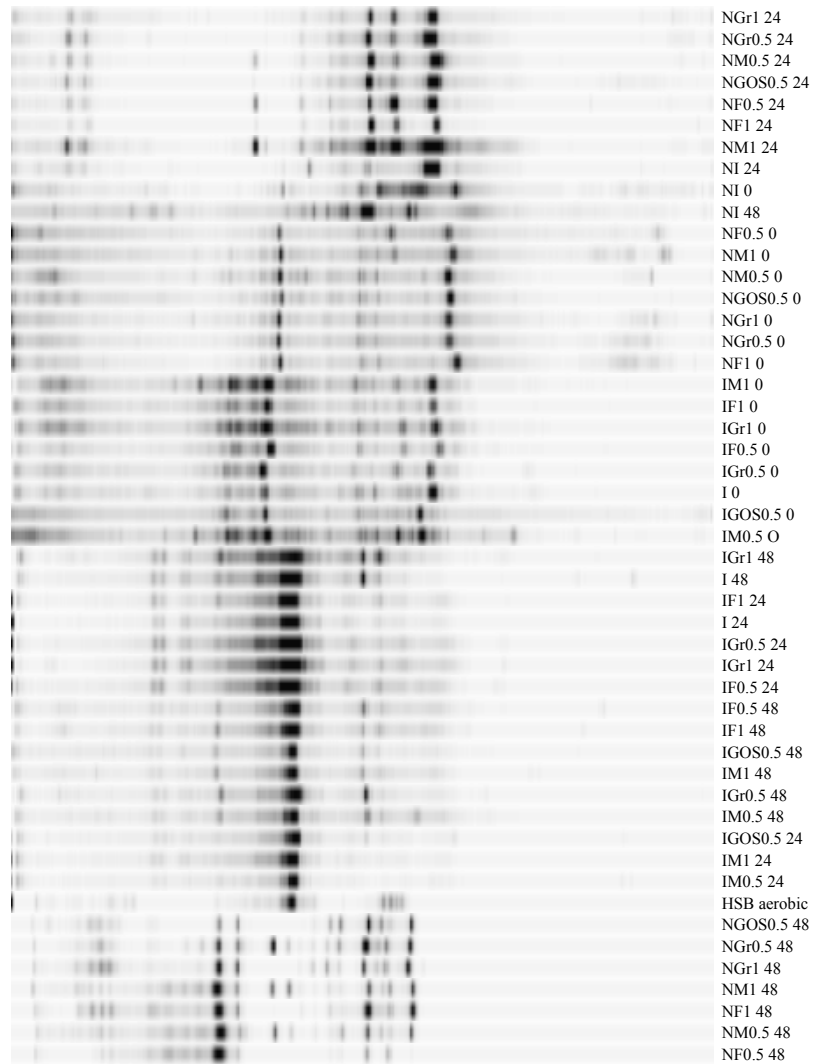
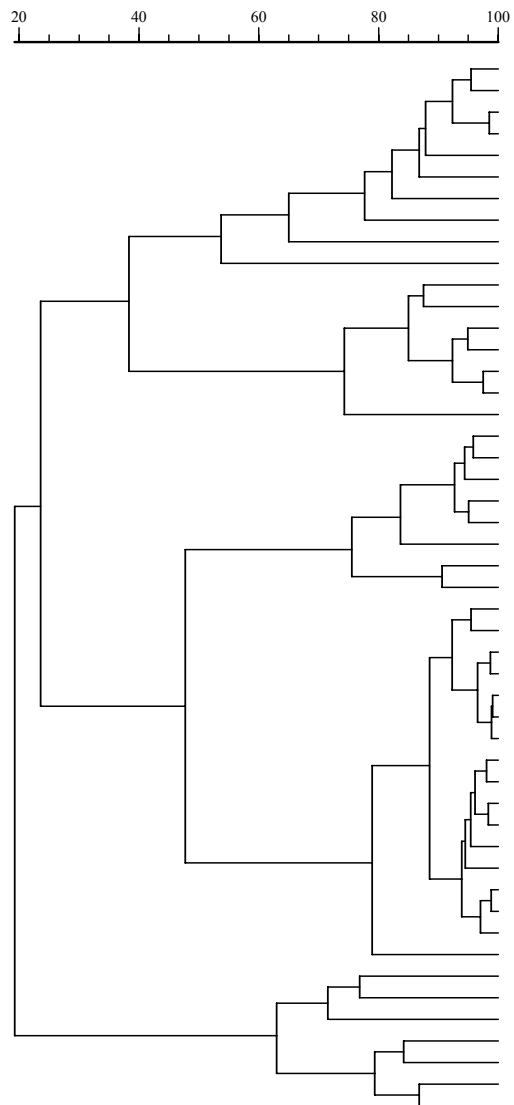
VFA production compared to the inoculated control; however, these differences disappeared after 48 h. There were no significant differences among the inoculated samples after 48 h ($P>0.05$).

DGGE analysis

The cluster analysis separated the samples by incubation time and inoculation status (Figure 2). The inoculated samples at 24 and 48 h were closely related ($SC>80\%$) to each other, with a single band dominating the communities. The 0-h incubation time point for the inoculated samples exhibited multiple bands and showed greater population diversity than the 24 and 48 h samples with. The dominant band detectable after 24 and 48 h of incubation was not visible in the 0-h samples. The 0-h inoculated samples had greater complexity than the 0-h non-inoculated samples, as evidenced by the greater number of bands. The non-inoculated samples had a greater diversity than the inoculated samples at 24 and 48 h as indicated by more bands. The only sample that did not cluster by incubation time and inoculation status was the non-inoculated 48-h sample that was most closely related to the non-inoculated 0-h sample. The non-inoculated samples were clustered as a function of incubation time with 0-h in one grouping and the 24-h and 48-h samples in another grouping. The 0-h samples had distinct microbial communities when compared to the 24- and 48-h samples ($SC<80\%$).

Each incubation time and inoculum level had at least one treatment that produced a distinct microbial community from the other treatments. For the 0-h non-inoculated treatments, the microbial community incubated with control diet and the 1% MOS diet

Figure 2. Denaturing gradient gel electrophoresis of bacterial 16S rDNA amplicons from hybrid striped bass intestinal contents. The bar above figure indicates percentage similarity coefficients. NI = non-inoculated + basal diet; NGr1 = non-inoculated + basal diet + 1% GroBiotic[®]-A; NGr0.5 = non-inoculated + basal diet + 0.5% GroBiotic[®]-A; NF1 = non-inoculated + basal diet + 1% FOS; NF0.5 = non-inoculated + basal diet + 0.5% FOS; NM1 = non-inoculated + basal diet + 2% mannanoligosaccharide; NM0.5 = non-inoculated + basal diet + 0.5% mannanoligosaccharide; NGOS0.5 = non-inoculated + basal diet + 0.5% galactooligosaccharide; I = inoculated + basal diet; IGr1 = inoculated + basal diet + 1% GroBiotic[®]-A; IGr0.5 = inoculated + basal diet + 0.5% GroBiotic[®]-A; IF1 = inoculated + basal diet + 1% FOS; IF0.5 = inoculated + basal diet + 0.5% FOS; IM1 = inoculated + basal diet + 2% mannanoligosaccharide; IM0.5 = inoculated + basal diet + 0.5% mannanoligosaccharide; IGOS0.5 = inoculated + basal diet + 0.5% galactooligosaccharide. The number indicates incubation time in hours.



was distinct from the other treatments. The communities incubated with 0.5% MOS and 0.5% GOS were distinct from the other treatments. This indicated that the starting communities were not identical even though all inocula came from a common source. After 24 h any differences in the inoculated samples seen at 0 h disappeared and all the samples were related (SC>85%). The non-inoculated samples still had distinct communities from those samples given the inoculum after 24 and 48 h of anaerobic incubation with about 62 and 80% SC, respectively. After 48-h non-inoculated samples, the FOS and MOS non-inoculated samples clustered together and the GroBiotic[®]-A and the GOS clustered together, while the basal sample was unique and clustered with the 0-h non-inoculated sample. The non-inoculated samples that contained 0.5% FOS and MOS were related (SC>80%) and distinct (SC<80%) from the non-inoculated samples that contained 1% FOS and MOS (SC>80%). The two non-inoculated Grobiotic[®]-A samples and the non-inoculated 0.5% GOS sample each contain a distinct microbial community (SC<80%).

Sequencing

The clones from the three major and shared non-inoculated bands all resembled the anaerobe *Clostridium perfringens* when processed through the Blast database (nr) database. The common band for the inoculated samples had a sequence closely resembling the anaerobe *Fusobacteria bacterium*.

Discussion

This study is the first to examine the production of VFAs *in vitro* by cultured hybrid striped bass intestinal digestive microflora. Acetate was produced in the highest concentrations and comprised 95 to 98% of VFA production after 24 h in inoculated samples but decreased to 68 to 80% of VFAs produced after 48 h. Acetate comprised 76-100% in the non-inoculated samples after 24 h and decreased to 61 to 68% after 48 h. These results are similar to what has been reported in some *in vivo* studies with fish (Smith et al. 1996; Mountfort et al. 2002). In the warm-temperate species *Medialuna californiensis* and two subtropical species, *Kyphosus bibibbus* and *K. vaigeinsis*, acetate production accounted for less than 20% of the VFAs produced while acetate production accounted for all of the VFA production in the cool-temperate species *Cebidichthys violaceus* (Kandel et al. 1994). *In vitro* acetate production after 48 h in the present study was approximately 4 to 5 times higher than in previous *in vivo* measurements (Smith et al. 1996; Mountfort et al. 2002) indicating that the culturable acetogenic species of microorganisms were more abundant and had more substrate available than *in vivo* communities. Smith et al. (1996) found that largemouth bass *Micropterus salmoides* had acetate concentrations of 33.5 mM in the GI tract in summer. Mountfort et al. (2002) examined temperate herbivorous fishes and found acetate concentrations ranging from 8.3 to 37.5 mM in the GI tracts of three temperate herbivorous species (*K. sydneyanus*, *Odax pullus*, and *Aplodactylus arctidens*).

Butyrate concentrations in the present study comprised about one third of VFA production after 48 h and were at least three times higher than detected in *in vivo* studies

in other laboratories. Butyrate was virtually undetectable in temperate freshwater species and three marine species (Smith et al. 1996; Mountfort et al. 2002); whereas, in tropical species butyrate comprised about 15-20% of the VFAs produced. Butyrate has been demonstrated to have beneficial effects on the host by decreasing pathogenicity of disease-causing bacteria (Van Immerseel et al. 2006) or by modulating leukocyte activity and increasing the innate immune response of the host (Nilsson et al. 2003; Raqib et al. 2006). In addition, the VFAs could be used as an energy source for the host. Tilapia has been shown to transport VFAs across the intestinal walls (Titus and Ahearn 1988; 1991) and thus the increased VFA production could be used as an energy source for the host fish.

Based on the variation of VFA production in the individual samples in this study, these results support the contention that the intestinal community varies from fish to fish. This VFA production variability also could be due to bacterial species not being uniformly distributed among samples or unique members of the intestinal community occurring in individual fish

Previous *in vivo* studies have evaluated the effects of GroBiotic®-A on performance and immune responses of hybrid striped bass. Li and Gatlin (2004; 2005) found that hybrid striped bass fed a diet containing 2% GroBiotic®-A had a significantly higher feed efficiency and significantly lower mortality when challenged with the bacterial pathogens *Streptococcus iniae* and *Mycobacterium marinum*. Innate immune responses in hybrid striped bass also tended to be increased in fish fed diets containing GroBiotic®-A or brewers yeast. Neutrophil oxidative radical anion production and

intracellular superoxide anion production by the head kidney tended to be greater and extracellular superoxide anion production was significantly greater in fish fed GroBiotic[®]-A compared to those fed the basal diet. However, changes to the microbial community were never examined.

In the current study, the addition of prebiotics did not change the anaerobic/facultative microbial community *in vitro* when compared to the microbial in the control diet. The inoculated samples had distinct microbial communities when compared to the non-inoculated samples indicating that the microbial community on the feed did not significantly contribute to the culturable GI tract community. The non-inoculated samples also had greater diversity 24 and 48 h inoculated samples indicating that the number of culturable species was greater in the feed than the hybrid striped bass GI tract. The dominant culturable species from the hybrid striped bass GI tract appeared to suppress the microbial diversity found on the feed. The lack of detectable change in the inoculated samples could be due to the lack of culturable microorganisms in the hybrid striped bass GI tract that are adapted to use β -linked carbohydrates or the community changes are below the detection level of DGGE. The present experiment only examined the culturable community associated with the GI tract of tank-reared hybrid striped bass. It has been estimated that only 5 to 20% of the species in the GI tract of mammals can be cultured using current media and culture methods (Moore and Holdeman 1974; Ward et al. 1990).

Results from the current study demonstrated that a single species of bacteria dominated the microbial community after being cultured for 24 and 48 h in an anaerobic

environment. The major band present in all 48-h samples (Fig. 2) was most likely *Fusobacteria bacterium*. *Fusobacteria* has butyrate as a major end product of fermentation (Holt et al. 1994), which is consistent with the increased butyrate production in the inoculated samples. The non-inoculated samples had much lower butyrate production and a dominant *Fusobacteria* band was lacking in these samples. The dominant species in the non-inoculated 48-h sample was *Clostridium perfringens*, which is a well known human pathogen (Johnson 1990). This pathogen did not appear to colonize the hybrid striped bass GI tract and appeared to be competitively excluded by the other culturable GI tract microbiota after 24 and 48 h of incubation. However, it is possible that other species having similar DNA patterns also may have been present. The banding pattern from the inoculated samples after 24 and 48 h of incubation were much less complex than previously reported from *in vivo* samples. Larval coho salmon (*Onchorhynchus kisutch*) were reported to have simple DGGE profiles consisting of only four bands with *Pseudomonas* spp. and *Aeromonas* spp. being the dominant species detected (Romero and Navarrete 2006). The initial (0 h) sample DGGE profiles from the present study were similar to the *in vivo* profiles that have been reported Coho salmon and haddock larvae *Melanogrammus aeglefinus* (Romero and Navarrete 2006; Plante et al. 2007). This difference is probably due to numerous species that cannot be cultured, but reside in the GI tract of a living host.

Pond et al. (2006) reported that the intestinal tract bacteria varied from fish to fish, thus complicating potential comparisons between treatments. The portion of the intestine where the sample was taken also can influence the bacterial community, with

the posterior sections of the gut typically having a more diverse microbial community (Moran et al. 2005). The uptake of a potential probiotic organism by haddock *Melanogrammus aeglefinus* larva was confirmed using DGGE (Plante et al. 2007). However, the changes in the microbial community related to diet and age were not analyzed and only certain microbial species were determined from each gel. Additional research using culture independent methods, metagenomics, and culturing the intestinal microorganisms are needed to more fully characterize the GI tract microbial community of fish as influenced by pro- and pre-biotic compounds.

The addition of prebiotics to the GI tract contents of hybrid striped bass did not alter the microbial community *in vitro*. Two previous *in vivo* studies conducted with GroBiotic®-A and hybrid striped bass indicate that there is some benefit for the hybrid striped bass, but a change in the culturable microbiota was not detected. These beneficial changes are noted by the increased growth performance and increased disease resistance of hybrid striped bass (Li and Gatlin 2004; 2005). One benefit could be the increased production of butyrate. This could lead to increased disease resistance and increased growth rate. Future studies need to be conducted to identify the microbial species involved and determine if there are any changes *in vivo*.

CHAPTER IV

THE EFFECTS OF GROBIOTIC[®]-A AND INULIN ON THE INTESTINAL MICROBIOTA AND GROWTH PERFORMANCE OF RED DRUM (*Sciaenops ocellatus*)

Synopsis

Two studies examined the effects of dietary supplementation of the prebiotics GroBiotic[®]-A and inulin on growth performance and the intestinal microbiota of the red drum *Sciaenops ocellatus*. In a second experiment, a secondary objective was to determine if the dietary prebiotics administered to fish in tanks sharing a common biofilter, sand filter and settling chamber, could affect fish not fed the prebiotic. The first experiment consisted of an 8-week feeding trial with fishmeal-based diets. Fifteen red drum were stocked per tank with an initial weight of 2.6 g. In the second study, red drum were stocked in a common water system with 15 fish (15.8 g initial average weight) in 110-L aquaria and concurrently six fish of the same origin were stocked in 110-L aquaria with individual biofilters (independent aquaria). A soybean meal/fish meal basal was supplemented with the prebiotics at 1% by dry weight and randomly assigned to each of three aquaria in the common water system and 3 independent systems and fed for 6 weeks. The fish fed the prebiotic supplemented diets had significantly lower whole-body protein compared to fish fed the basal diet. Red drum fed the inulin-supplemented diet had higher whole body moisture compared fish fed the basal diet. In the second experiment, the culture system significantly affected weight

gain, feed efficiency ratio and protein efficiency ratio. There were not any significant differences among treatments for feed efficiency or whole-body protein, lipid, moisture or ash among the treatments in the second trial. The microbial community was not affected by the addition of the prebiotics, revealed by the denaturing gradient gel electrophoresis (DGGE) analysis. The microbial community appears to be dominated by a single organism with very low diversity when compared to other livestock and fish species. Denaturing gradient gel electrophoresis of the microbial community in the filters of the independent tanks revealed that a diverse community was present, but the prebiotics did not have a detectable effect on the microbial community of the filters.

Introduction

The microbiota of the gastrointestinal (GI) tract plays important roles in the health and performance of the host (Fioramonti et al. 2003; Patterson and Burkholder 2003). Recently there has been increased interest in altering the intestinal microbiota for the benefit of the host by introducing beneficial bacteria into the diet, termed probiotics (Fioramonti et al. 2003) or adding non-degradable supplements to the diet that selectively stimulate the growth and/or activity of one or a limited number of beneficial bacterial species which are called prebiotics (Gibson and Roberfroid 1995). Probiotics have been studied in pigs (Sakata et al. 2003; Gardiner et al. 2004), chickens (Patterson and Burkholder, 2003) and humans (Fioramonti et al. 2003) as well as fishes (reviewed by Gatesoupe 1999; Gardiner et al. 2004; Irianto and Austin 2002; Vine et al. 2004). Prebiotics have been found to have beneficial effects in humans (reviewed by Manning

and Gibson 2005; Rastall 2004), poultry (Patterson and Burkholder 2003; Chung and Day 2004), and swine (Smiricky-Tjardes et al. 2003, Konstantinov et al. 2004). Reports from these studies reveal that prebiotics can modify the GI tract microbial community to enhance non-specific immune responses (Bailey et al. 1991), increase fermentation products (Smiricky-Tjardes et al. 2003), as well as improve mineral uptake (Bongers and van den Heuvel 2003) and livestock performance indices such as protein efficiency ratio and feed conversion ratio (Kirkpinar et al. 2004). These potential benefits of prebiotics have not been extensively investigated in fishes (Burr et al. 2005).

Prebiotics have received considerable attention from the terrestrial livestock industry as a way to improve disease resistance and to increase growth performance of the host organism. However, little attention has been given to prebiotics in aquaculture. To date there have been limited studies with fish evaluating prebiotics and other dietary constituents on microbiota of the GI tract. Linoleic acid and other dietary fatty acids, as well as dietary carbohydrate components, have been shown to alter the aerobic/facultative intestinal microbiota of Arctic charr *Salvelinus alpinus* using classical microbiological techniques (Ringø 1993; Ringø et al. 1998; Ringø 1999). When linoleic acid was supplemented in the diet of Arctic charr, the total viable counts from the GI tract aerobic/facultative microbial community were increased 10-fold as compared with fish fed a diet without linoleic acid (Ringø et al. 1998).

Li and Gatlin (2004; 2005) found that hybrid striped bass *Morone chrysops* x *M. saxatilis* fed a diet containing the prebiotic GroBiotic-A[®], a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products

had a significantly higher feed efficiency and lower mortality when challenged with the bacterial pathogens *Streptococcus iniae* and *Mycobacterium marinum*. Innate immune responses of hybrid striped bass also tended increase in fish fed diets containing GroBiotic-A[®] or brewers yeast. Neutrophil oxidative radical anion production from whole blood and intracellular superoxide anion production by the head kidney tended to be greater and extracellular superoxide anion production was significantly greater in hybrid striped bass fed GroBiotic-A[®] compared to those fed the basal diet. However, changes to the microbial community of the GI tract were never examined in these studies.

The purpose of the current study was to determine if Grobiotic[®]-A and inulin are effective prebiotics for red drum *Sciaenops ocellatus*. The supplements were evaluated in two growth trials and then the microbial community was assessed using denaturing gradient gel electrophoresis (DGGE).

Materials and Methods

Diet formulation and feeding trial 1 and 2

All diets for trial 1 were fishmeal-based diets and were formulated to contain 40% protein and 10% lipid and 14.6 kJ digestible energy/g (Table 7). Prebiotics were added at 1% by dry weight to fish basal diet replacing cellulose in the diet. The prebiotics evaluated included GroBiotic[®]-A (International Ingredient Corporation, St. Louis, MO.) (consisting of a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products), and inulin.

Table 7. Composition (g/100 g dry weight) of each the basal diet in experiment 1 and 2.

| | | Experiment 1 | Experiment 2 |
|--------------------------------------|----------|-----------------|-----------------|
| IFN | | | |
| Select Menhaden Meal ¹ | 5-02-009 | 57.8 | 27.1 |
| Soybean Meal, Dehulled | 5-04-612 | n/a | 35.5 |
| Dextrin ² | | 24.5 | 18.0 |
| Menhaden Oil ³ | 7-08-049 | 4.2 | 8.5 |
| Vitamin Premix ⁴ | | 3.0 | 3.0 |
| Mineral Premix ⁴ | | 4.0 | 4.0 |
| Carboxymethyl cellulose ² | | 2.0 | 2.0 |
| Cellulose ² | | 4.7 | 0.9 |
| Chromium III Oxide ⁵ | | n/a | 1.0 |
| <i>Analyzed Composition</i> | | | |
| (% dry weight) ⁶ | | | |
| Protein (%) | | 41.9 | 40.3 |
| Lipid (%) | | 10.0 | 10.7 |
| Gross energy (Kcal/kg) | | n/a | 4567 |

¹ Contained 73.8% protein and 10.0% lipid; Omega Protein Corporation, Inc., Abbeville, LA.

² USB Corporation, Cleveland, OH

³ Omega Protein Corporation, Inc., Reedville, VA

⁴ Moon and Gatlin (1991)

⁵ Sigma-Aldrich, St. Louis, MO

⁶ Means of duplicate analysis

All diets for trial 2 were formulated to contain 40% protein and 10% lipid, and to provide 14.6 kJ digestible energy/g. The diets were formulated to provide approximately 50% of the protein from fishmeal and 50% from soybean meal (Table 7). Each prebiotic was added at 1% by dry weight to the basal diet in place of cellulose. The prebiotics evaluated included GroBiotic[®]-A and inulin. Diets were cold pelleted, processed and stored according to established procedure (Webb and Gatlin 2003). These diets also contained chromic oxide as they also were used in a separate digestibility study.

Two separate feedings trial were conducted at the Texas A&M University Aquacultural Research and Teaching Facility. In the first feeding trial, 15 red drum (initially 2.6 ± 0.2 g each) were stocked into each aquarium and lasted 8 weeks. The system was composed of fifteen 110-L aquaria connected to a common sand filter and biological filter to maintain water quality. Water was brackish water (8 ‰) prepared from well water and a mixture of stock salt and commercial concentrated synthetic seawater. Temperature was maintained at 25°C by conditioning the ambient air and fish were subjected to a 12:12 light:dark photoperiod maintained by artificial lighting controlled by a timer. Dissolved oxygen was maintained close to saturation by blowing compressed air through air stones in each tank. The water quality of the systems was monitored periodically.

In feeding trial 2, the fish were stocked into either aquaria connected via a common water system or into aquaria each with independent biofiltration. The common water system was the same as used in feeding trial 1. The independent aquaria each had

individual filter pads for mechanical and biological filtration through which water was recirculated with submersible pumps. Fifteen red drum (initially 18.6 ± 0.1 g each) were stocked into each common-water aquaria and six red drum of the same origin were stocked into each independent-water aquarium. Fewer red drum were stocked into the independent tanks so that adequate water quality could be maintained throughout the 6-week feeding trial. Environmental and water conditions were the same as in trial 1 and the water quality of the systems was monitored periodically.

In feeding trial 1, triplicate tanks of red drum were randomly assigned one of the three diets and fed at a fixed percentage of their body weight divided into two daily feedings. The feedings initially started at 7% of their body weight and gradually decreased to 4% over the trial. The fish were weighed weekly and the feeding rate was adjusted accordingly to maintain a rate close to apparent satiation. The same experimental design was used in feeding trial 2, except the feeding rate was different initially starting at 4% of body weight and gradually decreased to 3%.

Four hours after feeding at the conclusion of feeding trial 2, the intestinal tracts of three red drum per tank were aseptically removed and the contents expressed by squeezing into a sterile microcentrifuge tube. The intestinal contents were then frozen at -20°C until denaturing gradient analysis could be performed.

Statistical analysis

At the end of each trial, weight gain, feed efficiency and protein efficiency ratio were subjected to analysis of variance and Duncan's multiple range test for comparison

using the Statistical Analysis System (SAS, 1985). Statistical significance was set at $P \leq 0.05$.

DNA isolation, PCR and denaturing gradient gel electrophoresis

To compare the GI tract microbial populations of fish fed the various diets in experiment 2, genomic DNA was isolated from the individual intestinal contents (0.2 ml to 0.5 ml) of 3 fish per aquaria with the Bio-Rad Aqua Pure DNA Isolation Kit (Bio-Rad, Hercules, CA) using the method supplied by the manufacturer with the following modifications. The pellets were suspended in 300 μ l of DNA lysis buffer. Twenty microliters of 20 mg/ml lysozyme was added and mixed with a sterile pestel. The solution was then incubated at 37°C for 2 h, centrifuged at 20,800 x g for 3 min and the supernatant removed and placed into a clean 1.5-ml microcentrifuge tube. Then 1.5 μ l of RNase (4 mg/ml) solution was added and the mixture was incubated at 37°C for 45 min.

PCR was conducted using the method of Hume et al. (2003) with bacteria-specific PCR primers to conserved regions flanking the variable V3 region of 16S rDNA. The primers (50 pmol of each primer; primer 2, 5'-ATTACC GCGGCTGCTGG-3'; primer 3 with a 40 base pair GC clamp (Muyzer et al., 1993) 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG GCAGCAG-3') were mixed with Jump Start Red-Taq Ready Mix (Sigma Chemical Company, St. Louis, MO) according to the manufacturer's instructions, 250 ng of pooled (83 ng/replicate) template DNA was added along with 10 μ g of bovine serum albumin

(BSA) to help stabilize the reaction. The PCR amplifications were preformed on a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). A touchdown PCR program was used to minimize artificial by-products. The program used was as follows: 1) denaturation at 94.9°C for 2 min; 2) denaturation at 94.0°C for 1 min; 3) annealing at 67°C for 45 s, -0.5°C per cycle (to minimize formation of artificial products) (Hume et al., 2003); 4) extension at 72°C for 2 min; repeat steps 2 to 4 for 17 cycles; 5) denaturation at 94°C for 1 min; 6) annealing at 58°C for 45 sec; 7) repeat steps 6 to 7 for 12 cycles; 8) extension at 72°C for 30 min; 9) 4°C final.

Denaturing gradient gel electrophoresis (DGGE) was run following the method of Hume et al. (2003) as modified from Muyzer et al. (1993). The amplicons were separated on 8% polyacrylamide gels [(vol/vol) acrylamide-bisacrylamide ratio of 37.5:1 (Bio-Rad, Richmond, CA)] with a 30 to 60% urea-formamide gradient (100% denaturing 7M urea and 40% formamide) using a Dcode System (Bio-Rad, Hercules, CA). The amplicons were mixed with an equal volume of 2X loading buffer [0.05% (wt/vol) bromophenol blue; 0.05% (wt/vol) xylene cyanol; and 70% (vol/vol) glycerol] and 7 µl was loaded into each sample well (16-well comb). The gels were run at 60 volts for 17 hours in 0.5X TAE (20 mM Tris (pH 7.4); 10 mM sodium acetate; 0.5 M EDTA; Bio-Rad, Hercules, CA) at 59°C. Gels were stained for 30 min with SYBR Green I (USA Amersham Life Sciences, Cleveland, OH) diluted 1:10,000. The fragment analysis pattern relatedness was determined with Molecular Analysis Fingerprinting software (v 1.6; Bio-Rad, Hercules, CA). This analysis is based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages (UPGMA) for

clustering. Comparisons between sample band patterns were expressed as a percentage similarity coefficient (%SC).

Results

Trial 1

Weight gain, feed efficiency (FE), and protein efficiency ratio (PER) did not vary among the treatments (Table 8). Weight gain ranged from 119.1 to 132.6% with the higher weight gain for the fish fed the prebiotic supplemented diets. Feed efficiency ranged from 0.66 to 0.70, again with the fish fed the prebiotic-supplemented diets having the higher FE. Protein efficiency ratio ranged from 1.77 to 1.89.

The whole-body moisture and protein varied significantly ($P \leq 0.05$), while whole-body ash and lipid did not vary among the treatments (Table 9). Fish fed the inulin-supplemented diet had significantly higher whole-body moisture when compared to fish fed the basal diet ($P \leq 0.05$). The fish fed the basal diet had significantly ($P \leq 0.05$) higher whole-body protein when compared to the fish fed the GroBiotic[®]-A or inulin supplemented diets.

Trial 2

The prebiotic supplemented diets did not enhance growth after 6 weeks (Table 10). The percent weight gain over the 6-week trial ranged from 160 to 192% with the higher weight gains tending to be in the common water system. The fish in the common water system had more uniform growth while the fish in the individual tanks showed greater variation.

Table 8. Growth performance of red drum fed the basal and experimental diets for experiment 1.¹

| Diet | weight gain (% increase) | FE (g gain/g fed) | PER (g gain/ g protein fed) |
|--------------------------------------|-----------------------------|----------------------|--------------------------------|
| Basal | 1190.8 ± 41.5 | 0.66 ± 0.01 | 1.77 ± 0.02 |
| Basal + 1% GroBiotic [®] -A | 1326.1 ± 145.3 | 0.70 ± 0.04 | 1.89 ± 0.11 |
| Basal + 1% inulin | 1322.8 ± 132.6 | 0.67 ± 0.08 | 1.82 ± 0.22 |
| Anova P ² | 0.3158 | 0.6467 | 0.6467 |

¹ values represent means ± standard deviation of three replicate groups. Initial fish weight is 2.6 ± 0.2 g/fish (mean ± one standard deviation).

² Significance probability associated with the F statistic. Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on Duncan's multiple range test.

Table 9. Whole-body composition of red drum fed the basal and experimental diets for experiment 1.¹

| Diet | Ash (%) | Moisture (%) | Protein (%) | Lipid (%) |
|--------------------------------------|-------------|----------------------------|---------------------------|-------------|
| Basal | 4.70 ± 0.78 | 73.23 ± 1.17 ^B | 18.4 ± 1.01 ^A | 4.95 ± 0.28 |
| Basal + 1% GroBiotic [®] -A | 4.11 ± 0.27 | 74.10 ± 0.26 ^{AB} | 16.44 ± 0.92 ^B | 4.90 ± 0.76 |
| Basal + 1% inulin | 3.99 ± 0.16 | 75.20 ± 0.15 ^A | 16.56 ± 0.45 ^B | 4.23 ± 0.20 |
| Anova P ² | 0.02346 | 0.0366 | 0.0481 | 0.2078 |

¹ Values represent means ± standard deviation of three composite samples of three fish from each of three replicate groups.

² Significance probability associated with the F statistic. Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on Duncan's multiple range test.

Table 10. Growth performance of red drum fed the basal and experimental diets for trial 2.¹

| Diet | weight gain (% increase) | Feed efficiency ratio (g gain/g fed) | Protein efficiency ratio (g gain/ g protein fed) |
|--------------------------------------|-----------------------------|---|---|
| Common water System | | | |
| Basal | 191.64 ± 7.36 ^A | 0.72 ± 0.01 | 1.99 ± 0.02 ^A |
| Basal + 1% GroBiotic [®] -A | 186.15 ± 8.87 ^{AB} | 0.70 ± 0.04 | 1.88 ± 0.10 ^{AB} |
| Basal + 1% inulin | 188.77 ± 8.22 ^{AB} | 0.71 ± 0.02 | 1.95 ± 0.03 ^A |
| Independent Aquaria | | | |
| Basal | 168.95 ± 7.00 ^{AB} | 0.63 ± 0.02 | 1.75 ± 0.05 ^{AB} |
| Basal + 1% GroBiotic [®] -A | 159.50 ± 32.33 ^B | 0.60 ± 0.05 | 1.58 ± 0.12 ^B |
| Basal + 1% inulin | 186.33 ± 9.20 ^{AB} | 0.68 ± 0.13 | 1.85 ± 0.37 ^{AB} |
| One-way Anova P ² | 0.1257 | 0.1863 | 0.1045 |
| Two-way Anova P ³ | | | |
| Diet | 0.2812 | 0.5399 | 0.2094 |
| System | 0.0326 | 0.0252 | 0.0205 |
| Diet × System | 0.3638 | 0.5654 | 0.6008 |

¹ Values represent means ± standard deviation of three replicate groups. Initial fish weight is 18.6 ± 0.1 g/fish (mean ± one standard deviation).

² Significance probability associated with the F statistic. Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on Duncan's multiple range test.

³ Significance probability associated with the F statistic

Feed efficiency did not vary among systems or diets for this six-week trial (Table 11). Feed efficiency (FE) ranged from 0.70 to 0.72 in the common water system and 0.63 to 0.68 in the independent tank system. Protein efficiency ratio (PER) ranged from 1.88 to 1.99 in the common water system and 1.58 to 1.85 in the independent tank system. The system significantly ($P \leq 0.05$) influenced weight gain, FE and PER for trial 2. Proximate composition analysis of the fish carcass did not vary among treatment or system (Table 10).

Denaturing gradient electrophoresis revealed that in red drum a single species, or species that contained chemically equivalent DNA, dominated the intestinal tract of all samples regardless of dietary treatment (Figure 3). The filter samples were more diverse and did not contain a dominant single species (Figure 4). The three filters from aquaria containing fish fed the basal + 1% inulin all clustered together and were related ($SC > 80\%$). The filters from aquaria containing fish fed the basal diet and the basal diet + 1% GroBiotic®-A diets were overlapping with each other filters. There was not a pattern to the clustering of these two treatments.

Discussion

In trial 1, the effects of prebiotics on growth were examined. In trial 2, the effects of the prebiotics on the intestinal microbiota and growth were assessed along with the effects of a common water system on the microbiota and growth. Red drum fed the prebiotic-supplemented diets did not have significantly greater weight gain, FER, or protein efficiency ratio compared to the basal diet in both trials. The growth rate for

Table 11. Whole-body composition of red drum in experiment 2.¹

| Diet | Ash (%) | Moisture (%) | Protein (%) | Lipid (%) |
|------------------------------|-------------|--------------|--------------|-------------|
| Common water System | | | | |
| Basal | 3.86 ± 0.19 | 74.96 ± 0.54 | 17.03 ± 0.34 | 3.53 ± 0.53 |
| Basal + 1% GroBiotic®-A | 4.21 ± 0.33 | 75.38 ± 1.07 | 16.64 ± 1.16 | 3.65 ± 0.28 |
| Basal + 1% inulin | 4.44 ± 0.46 | 75.57 ± 1.01 | 16.81 ± 0.74 | 3.21 ± 0.22 |
| Independent Aquaria | | | | |
| Basal | 4.65 ± 0.24 | 74.30 ± 1.05 | 17.78 ± 0.04 | 3.94 ± 1.00 |
| Basal + 1% GroBiotic®-A | 4.21 ± 0.16 | 74.82 ± 0.92 | 16.72 ± 0.76 | 3.90 ± 1.03 |
| Basal + 1% inulin | 4.36 ± 0.63 | 74.37 ± 1.10 | 17.15 ± 1.09 | 3.68 ± 0.61 |
| Anova P ² | 0.3875 | 0.5729 | 0.7169 | 0.7998 |
| Two-way Anova P ³ | | | | |
| Diet | 0.7100 | 0.7843 | 0.4639 | 0.6704 |
| System | 0.3090 | 0.1057 | 0.3894 | 0.2670 |
| Diet × System | 0.1849 | 0.8254 | 0.8078 | 0.9558 |

¹ Values represent means ± standard deviation of three composite samples of 3 fish from each of three replicate groups.

² Significance probability associated with the F statistic. Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on Duncan's multiple range test.

³ Significance probability associated with the F statistic

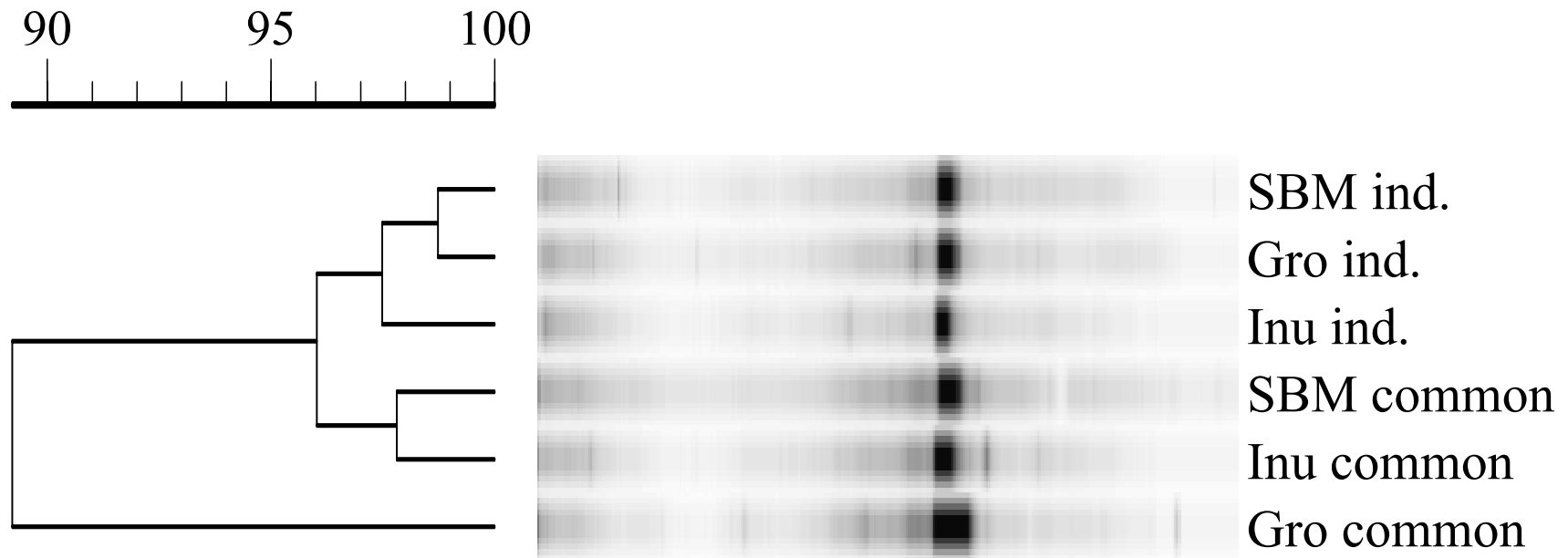


Figure 3. Denaturing gradient gel electrophoresis of bacterial 16S rDNA amplicons from red drum intestinal contents. The bar above figure indicates percentage similarity coefficients. SBM ind. = fish fed the basal diet in the independent aquaria system; Gro ind = fish fed the basal diet + 1% GroBiotic[®]-A in the independent aquaria system; Inu Ind. = fish fed the basal diet + 1% inulin in the independent aquaria system; SBM common = fish fed the basal diet in the common water; Gro common = fish fed the basal diet + 1% GroBiotic[®]-A in the common water system; Inu common = fish fed the basal diet + 1% inulin in the common water system

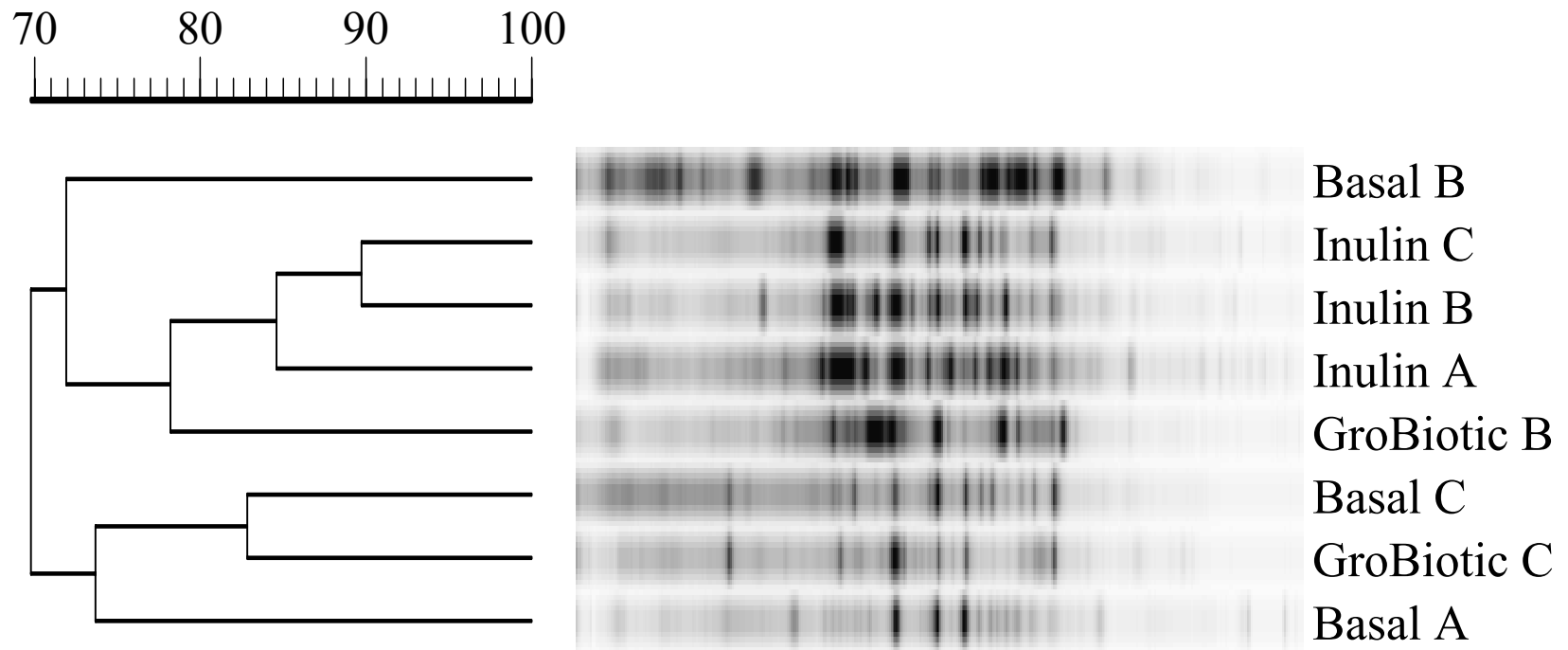


Figure 4. Denaturing gradient gel electrophoresis of bacterial 16S rDNA amplicons from film deposited on the biofilters of the independent aquaria *in vivo*. The bar above figure indicates percentage similarity coefficients. The letters after the treatments indicate individual aquaria.

both trials was similar when fish of similar initial size were compared (data not shown). The FER and PER also were similar between the two trials. These similarities indicate that unprocessed soybean meal can replace some of the fishmeal in the diet. In previous studies up to 95% of crude protein was supplied by soybean meal without a decrease in weight gain and feed efficiency ratio (McGoogan and Gatlin 1997). The FER in both trials in the current study was lower than values reported for red drum fed a red drum muscle- or fishmeal-based diet in previous experiments (Webb and Gatlin 2003; Whiteman and Gatlin 2005; Li et al. 2006). The lower FER indicates that the protein in the soybean meal was not as digestible and the growth of juvenile red drum was lower when compared to other feeding trials. The prebiotics did not seem to improve the performance of juvenile red drum in either feeding trial and is in contrast to what was observed with sub-adult red drum, in which fish fed these prebiotic-supplemented diets showed a higher protein digestibility when compared to the basal diet (Burr et al. 2007). The increased digestibility values in sub-adult red drum could be due to a lack of a complex microbial community being established in the GI tract of such immature fish that have been medicated. The protein efficiency ratio values in the present feeding trials were slightly higher than was reported for fish fed a diet containing 37% protein (Burr et al. 2006). This absence of an increased PER also indicates that the prebiotics are not altering the microbiota to make amino acids or the protein available for the juvenile fish. Hybrid striped bass fed similar diets did not have increased weight gain, feed efficiency, or protein efficiency ratio, but the intestinal microbial community was distinctly different when fish were fed diets supplemented with inulin or GroBiotic®-A

(unpublished data). The intestinal microbial community of the hybrid striped bass was more complex, with 4-6 detectable DGGE bands, compared to the single DGGE band detected for red drum.

The intestinal tracts of the red drum were dominated by a single band indicating that a single species or several species with chemically equivalent DNA were present. This could be due to the addition copper to the water as a preventative measure against *Amylodinium*. GroBiotic[®]-A and inulin were evaluated *in vitro* with the culturable anaerobic/facultative anaerobic microbial community of red drum and the addition of GroBiotic[®]-A to the diet altered the microbial community when compared to a basal diet (Burr et al. 2006). The dendrograms from the *in vitro* study showed a diverse culturable community with dominant bands being identified as the facultative anaerobes *Lactococcus lactis* and an *Aeromonas* sp. The DGGE profile from the current *in vivo* experiment is very simple when compared to other species (Pond et al. 2006; Ring et al. 2006; Plante et al. 2007). It is possible that the intestinal microbial community of juvenile red drum kept in a closed recirculating system did not have the chance to mature and thus provide the diversity found at later life stages (Burr et al. 2007). The DGGE profiles of sub-adult fish (approximately 500 g) fed these same diets in a previous experiment (Burr et al. 2007) were much more complex and were not dominated by a single species. The DGGE banding pattern of the sub-adult fish also showed that the microbial communities of the fish fed GroBiotic[®]-A and inulin differed from that of fish fed the basal diet (Burr et al. 2007). It is possible that different results might have been

obtained with fish that had a mature microbial community as would presumably be present in larger fish.

The red drum in the present study had a very simple DGGE profile when compared to haddock larvae *Melanogrammus aeglefinus*, coho salmon *Oncorhynchus kisutch*, and rainbow trout *Oncorhynchus mykiss* (Heikkinen et al. 2006, Romero and Navarrete 2007, Plante et al. 2007). The reported DGGE profiles for coho salmon contained a minimum of four bands, while the current study had only a single dominant band. It is possible that other bands were present at levels much lower than the dominant band, but it is unlikely that these species would play a significant role in the microbial intestinal tract community. Even in juvenile rainbow trout there was a higher microbial diversity as detected by length-heterogeneity analysis of PCR amplified 16S rDNA compared to the diversity detectable in the present study (Heikkinen et al. 2006).

This is the first study to examine the microbial populations of the fish and biofilters to consider the potential influence of dietary prebiotics in a common water system compared to individual aquaria. GroBiotic[®]-A and inulin did not influence the intestinal microbial communities and did not increase growth of the red drum. The intestinal microbiota of the juvenile red drum might not have included all of the members that a more mature fish would have and thus the influence of the prebiotic compounds was not detected. The inclusion of inulin in the diets did seem to influence the microbial populations associated with the filters, but did not confer any detectable benefit to the red drum in the aquaria. Measured features of water quality did not vary among the independent aquaria and no advantage was detectable for having filters

containing one community or another community. The performance of fish was affected by the system possibly due to temporarily lower water quality in the independent aquaria, such as high levels of ammonia that is removed before detection or the combination of prebiotics altered the microbial community of the common biofilter that conferred an advantage to fish in the common system. The prebiotic-supplemented diets did not alter the intestinal microbiota of the red drum and did not confer enhanced performance in a closed recirculating system.

CHAPTER V

EFFECTS OF THE PREBIOTICS GROBIOTIC[®]-A, INULIN,
MANNANOLIGOSACCHARIDE, AND GALACTO-OLIGOSACCHARIDE ON THE
INTESTINAL MICROBIOTA AND GROWTH PERFORMANCE OF HYBRID
STRIPED BASS (*Morone chrysops* x *M. saxatilis*)

Synopsis

An 8-week feeding trial evaluated the effects of dietary prebiotic supplements, GroBiotic[®]-A, inulin, mannanoligosaccharide (MOS), and galacto-oligosaccharide (GOS), on growth performance and intestinal microbial composition of hybrid striped bass. A soybean meal/fish meal diet, with approximately 50% of the protein supplied by each was supplemented with either GroBiotic[®]-A, inulin, MOS, or GOS. Each diet was fed to juvenile hybrid striped bass in triplicate 110-L aquaria for 8 weeks. At the end of the feeding trial intestinal-tract content-samples were taken from three fish per aquaria for denaturing gradient gel electrophoresis (DGGE). Weight gain, feed efficiency ratio, protein efficiency ratio, and whole-body ash, moisture and lipid did not vary among fish fed the various dietary treatments. Fish fed the GroBiotic[®]-A-supplemented diet had significantly ($P \leq 0.05$) greater whole-body protein than those fed the basal diet. DGGE revealed that all of the prebiotics produced a unique microbial community in the intestinal tract of hybrid striped bass compared to fish fed the basal diet. The microbial

community of the fish fed the GOS-supplemented diet also was different from that of hybrid striped bass fed the other prebiotics.

Introduction

The microbiota of the gastrointestinal (GI) tract plays a role of increasingly evident importance in the health and performance of the host (Fioramonti et al. 2003; Patterson and Burkholder 2003). Recently there has been increased interest in manipulating the GI tract microbial community to enhance beneficial bacteria. There are two general approaches used to modify the GI tract bacteria. One involves feeding probiotics, which are viable microorganisms that are added to the diet to benefit the host (Fioramonti et al., 2003). Probiotics have been studied in pigs (Sakata et al. 2003, Gardiner et al. 2004), chickens (Patterson and Burkholder 2003) and humans (Fioramonti et al. 2003) as well as fishes (reviewed by Gatesoupe 1999; Gardiner et al. 2000; Irianto and Austin 2002; Vine et al. 2004). The second approach for modifying the GI tract microbial community is the addition of prebiotics to the diet. Prebiotics are defined as “nondigestable food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon” (Gibson & Roberfroid 1995). Prebiotics have been found to have beneficial effects in humans (reviewed by Manning & Gibson 2005; Rastall 2004), poultry (Patterson and Burkholder, 2003, Chung and Day 2004), and swine (Smiricky-Tjardes et al. 2003; Konstantinov et al. 2004). Reports from these studies reveal that prebiotics can modify the GI tract microbial community to enhance non-

specific immune responses (Bailey et al. 1991), increase fermentation products (Smiricky-Tjardes et al. 2003), improve mineral uptake (Bongers & van der Heuvel 2003) as well as enhance livestock performance indices such as protein efficiency ratio and feed conversion ratio (Kirkpinar et al. 2004). Smiricky-Tjardes et al. (2003) demonstrated that dietary transglacto-oligosaccharide increased the concentrations of the volatile fatty acids (VFAs) propionate and butyrate in the small intestine of swine. Prebiotics such as oligofructose have been reported to increase bioavailability of glucose and trace elements in the diet (Breves et al. 2001; Bongers & van der Heuvel 2003). Oligofructose also has been shown to increase feed efficiency and weight gain in broiler chicks, while mixed results have been seen in pigs (Flickinger et al. 2003). Prebiotics have received considerable attention from the terrestrial livestock industry as a way to improve disease resistance. These potential benefits of prebiotics have not been thoroughly investigated in fishes (Burr et al. 2005).

In fishes, limited studies have investigated the effects of nutrients such as linoleic acid and other fatty acids, as well as carbohydrate components, on the aerobic/facultative intestinal microbiota of Arctic charr *Salvelinus alpinus*, using classical microbiological techniques (Ringø 1993; Ringø et al. 1998; Ringø 1999). When linoleic acid was supplemented in the diet of Arctic charr, the total viable counts from the GI tract aerobic/facultative microbial community were increased 10-fold as compared with fish fed a diet without linoleic acid (Ringø et al. 1998). More recently, the prebiotic GroBiotic[®]-A was found to significantly increase feed efficiency and reduce mortality of hybrid striped bass when challenged with the bacterial pathogens *Streptococcus iniae*

and *Mycobacterium marinum* (Li and Gatlin 2004, 2005). Neutrophil oxidative radical anion production from the whole blood and intracellular superoxide anion production by the head kidney tended to be greater and extracellular superoxide anion production was significantly greater in hybrid striped bass fed GroBiotic[®]-A compared to those fed the basal diet. However, changes to the microbial community of the GI tract were not examined in these studies. The current study examined the effects of four prebiotics--GroBiotic[®]-A, inulin, mannanoligosaccharide (MOS), and galactooligosaccharide (GOS)--on growth performance and the intestinal microbial community of hybrid striped bass cultured in a closed recirculating system.

Methods and Materials

Diet formulation

All diets were formulated to contain 40% protein, 10% lipid, and 14.6 kJ digestible energy/g. The diets were formulated to have approximately 50% of the protein supplied by fishmeal and the remainder provided by soybean meal (Table 12). Prebiotics were added at 1% by weight to the basal diet replacing some of the cellulose. The prebiotics added were GroBiotic[®]-A (consisting of a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products), inulin, MOS, and GOS. Diets were prepared and as previously described (Webb and Gatlin 2003).

Table 12. Diet formulation (% dry weight basis) and proximate analysis

| Ingredient | IFN | Basal Diet | GroBiotic®-A | Inulin | MOS | GOS |
|--|----------|------------|--------------|--------|-------|-------|
| Menhaden fishmeal ¹ | 5-02-009 | 27.10 | 27.10 | 27.10 | 27.10 | 27.10 |
| Soybean Meal | 5-04-612 | 35.50 | 35.50 | 35.50 | 35.50 | 35.50 |
| Dextrin ² | | 16.60 | 16.60 | 16.60 | 16.60 | 16.60 |
| Menhaden Oil ³ | 7-08-049 | 7.58 | 7.58 | 7.58 | 7.58 | 7.58 |
| Vitamin Premix ⁴ | | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| Mineral Premix ⁴ | | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 |
| CMC ² | | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| GroBiotic®-A ⁵ | | n/a | 1.00 | n/a | n/a | n/a |
| MOS ⁶ | | n/a | n/a | n/a | 1.00 | n/a |
| Inulin ⁷ | | n/a | n/a | 1.00 | n/a | n/a |
| GOS ⁸ | | n/a | n/a | n/a | n/a | 1.00 |
| Celufil ² | | 4.22 | 3.22 | 3.22 | 3.22 | 3.22 |
| Analyzed Composition (% dry weight) ⁹ | | | | | | |
| Protein (%) | | 39.13 | 38.51 | 39.06 | 39.07 | 39.32 |
| Lipid (%) | | 11.06 | 11.30 | 11.20 | 12.31 | 12.44 |
| Ash (%) | | 11.36 | 10.84 | 10.96 | 11.33 | 10.53 |

¹ Contained 73.8% protein and 10.0% lipid; Omega Protein Corporation, Inc., Abbeville, AL.

² USB Corporation, Cleveland, OH

³ Omega Protein Corporation, Inc., Reedville, VA

⁴ Moon and Gatlin (1991)

⁵ International Ingredient Corporation, St. Louis, MO

⁶ Alltech Inc., Nicholasville, KY

⁷ Encore Technologies LLC, Plymouth, MN

⁸ Friesland Foods Domo, Zwolle, Netherlands

⁹ means of two analysis

The 8-week feeding trial took place at the Texas A&M University Aquacultural Research and Teaching Facility. Hybrid striped bass were stocked into 110-l aquaria connected to a common sand filter and biological filter to maintain optimal water quality. Water in the system was prepared from well water and a mixture of stock salt and commercial concentrated synthetic seawater to provide brackish water at 3 ‰. Temperature was maintained at 25°C by conditioning the ambient air and fish were subjected to a 12:12 light:dark photoperiod maintained by artificial lighting controlled by a timer. Dissolved oxygen was maintained close to saturation by blowing compressed air through air stones in each aquarium. The water quality of the systems was monitored periodically.

Ten juvenile hybrid striped bass (initially 344.4 ± 1.1 g per group) were stocked into each aquarium. Triplicate aquaria of hybrid striped bass were randomly assigned one of the five diets and fed at a fixed percentage of body weight divided into two daily feedings. The feeding rate initially started at 2.5% of body weight and gradually decreased to 1.5% over the trial. The fish were weighed weekly and the feeding rate adjusted according to biomass to maintain a rate close to apparent satiation. At the end of the trial, weight gain, feed efficiency ratio and protein efficiency ratio were computed. Three fish were collected from each tank for subsequent measurement of whole-body composition according to established procedures (Webb & Gatlin 2003).

Intestinal tract sampling, DNA isolation, PCR and denaturing gradient gel electrophoresis

Four hours after the final feeding, the intestinal tracts of three hybrid striped bass per aquarium were aseptically removed and the contents expressed by squeezing into a sterile microcentrifuge tube. The samples from three fish per aquarium were pooled into one composite sample (three replicates for each treatment and frozen at -20°C until denaturing gradient gel electrophoresis (DGGE) analysis could be preformed.

To compare the GI tract microbial populations of fish fed the various diets, genomic DNA was isolated from the individual intestinal contents (0.2 to 0.5 ml) with the Bio-Rad Aqua Pure DNA Isolation Kit (Bio-Rad, Hercules, CA) using the method supplied by the manufacturer with the following modifications. The pellets were suspended in 300 µl of DNA lysis buffer. Twenty µl of 20 mg/ml lysozyme were added and mixed with a sterile pestel. The solution was then incubated at 37°C for 2 h centrifuged at 20,800 x g for 3 min and the supernatant removed and placed into a clean 1.5-ml microcentrifuge tube. A 1.5-µl aliquot of RNase (4 mg/ml) solution was subsequently added and the mixture was incubated at 37°C for 45 min.

PCR was conducted using the method of Hume et al. (2003) with bacteria-specific PCR primers to conserved regions flanking the variable V3 region of 16S rDNA. The primers (50 pmol of each primer; primer 2, 5'-ATTACC GCGGCTGCTGG-3'; primer 3 with a 40 base pair GC clamp (Muyzer et al. 1993) 5'-CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG GCAGCAG-3') were mixed with Jump Start Red-Taq Ready Mix (Sigma Chemical

Company, St. Louis, MO) according to the manufacturer's instructions, 250 ng of pooled (83 ng/replicate) template DNA was added along with 10 µg of bovine serum albumin (BSA) to help stabilize the reaction. The PCR amplifications were performed on a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). A touchdown PCR program was used to minimize artificial by-products. The program used was as follows: 1) denaturation at 94.9°C for 2 min; 2) denaturation at 94.0°C for 1 min; 3) annealing at 67°C for 45 s, -0.5°C per cycle (to minimize formation of artificial products) (Hume et al., 2003); 4) extension at 72°C for 2 min; repeat steps 2 to 4 for 17 cycles; 5) denaturation at 94°C for 1 min; 6) annealing at 58°C for 45 sec; 7) repeat steps 6 to 7 for 12 cycles; 8) extension at 72°C for 30 min; 9) 4°C final.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was run following the method of Hume et al. (2003) as modified from Muyzer et al. (1993). The amplicons were separated on 8% polyacrylamide gels [(vol/vol) acrylamide-bisacrylamide ratio of 37.5:1 (Bio-Rad, Richmond, CA)] with a 30% to 60% urea-formamide gradient (100% denaturing 7M urea and 40% formamide) using a Dcode System (Bio-Rad, Hercules, CA). The amplicons were mixed with an equal volume of 2X loading buffer [0.05% (wt/vol) bromophenol blue; 0.05% (wt/vol) xylene cyanol; and 70% (vol/vol) glycerol] and 7 µl was loaded into each sample well (16-well comb). The gels were run at 60 volts for 17 hours in 0.5X TAE (20 mM Tris (pH 7.4); 10 mM sodium acetate; 0.5 M

EDTA; Bio-Rad, Hercules, CA) at 59°C. Gels were stained for 30 min with SYBR Green I (USA Amersham Life Sciences, Cleveland, OH) diluted 1:10,000.

DGGE Analysis

The fragment analysis pattern relatedness was determined with Molecular Analysis Fingerprinting software (v 1.6; Bio-Rad, Hercules, CA). This analysis is based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages (UPGMA) for clustering. Comparisons between sample band patterns were expressed as a percentage similarity coefficient (%SC).

Results

Hybrid striped bass fed the various diets had similar weight gain which ranged from 151% to 155% of initial weight (Table 13). Feed efficiency ratio (FER) also did not vary among treatments, ranging from 0.65 to 0.78 (Table 1). Protein efficiency ratio (PER) values did not differ statistically among treatments, ranging from 1.81 to 2.11 (Table 13). Whole-body ash, moisture and lipid composition of fish did not vary due to supplementation of prebiotics; however, fish fed the GroBiotic[®]-A-supplemented diet had significantly higher percentage of whole-body protein (18.6%) when compared to fish fed the basal diet (17.5%) (Table 14).

The intestinal microbial community of fish fed the basal diet was unique when compared to those of fish fed the prebiotic-supplemented diets (SC < 50%) (Figure 5). The microbial community of fish fed the GOS-supplemented diets also was distinctly

Table 13. Growth performance of hybrid striped bass fed the basal and experimental diets.¹

| Diet | % weight gain | Feed efficiency ratio (g gain/g fed) | Protein efficiency ratio (g gain/ g protein fed) |
|---------------------------|---------------|---|---|
| Basal | 153 ± 2.77 | 0.65 ± 0.05 | 1.81 ± 0.05 |
| GroBiotic [®] -A | 154 ± 8.21 | 0.73 ± 0.03 | 1.99 ± 0.03 |
| inulin | 152 ± 7.05 | 0.68 ± 0.06 | 1.88 ± 0.06 |
| MOS | 154 ± 2.62 | 0.74 ± 0.11 | 2.03 ± 0.11 |
| GOS | 151 ± 5.88 | 0.78 ± 0.07 | 2.11 ± 0.07 |
| Anova P | 0.9301 | 0.2801 | 0.3600 |

¹ Values represent means ± standard deviation of three replicate groups.

² Significance probability associated with the F statistic. Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on Duncan's multiple range test.

Table 14. Whole-body composition of hybrid striped bass fed the basal and experimental diets.¹

| Diet | Ash (%) | Moisture (% of fresh weight) | Protein (%) | Lipid (%) |
|---------------------------|-------------|------------------------------|----------------------------|-------------|
| Basal | 4.49 ± 0.52 | 67.93 ± 0.51 | 17.53 ± 0.80 ^B | 7.99 ± 0.49 |
| GroBiotic [®] -A | 4.94 ± 0.43 | 66.21 ± 1.03 | 18.64 ± 0.59 ^A | 8.02 ± 0.70 |
| Inulin | 4.54 ± 0.48 | 67.89 ± 0.75 | 17.44 ± 0.50 ^B | 8.27 ± 0.44 |
| MOS | 4.69 ± 0.18 | 67.90 ± 0.22 | 17.13 ± 0.30 ^B | 8.31 ± 0.48 |
| GOS | 4.40 ± 0.35 | 68.00 ± 2.60 | 17.96 ± 0.50 ^{AB} | 7.75 ± 2.06 |
| Anova P | 0.8589 | 0.4417 | 0.0599 | 0.9608 |

¹ Values represent means ± standard deviation of three replicate groups.

² Significance probability associated with the F statistic. Values in a column that do not have the same superscript are significantly different at $P \leq 0.05$ based on Duncan's multiple range test.

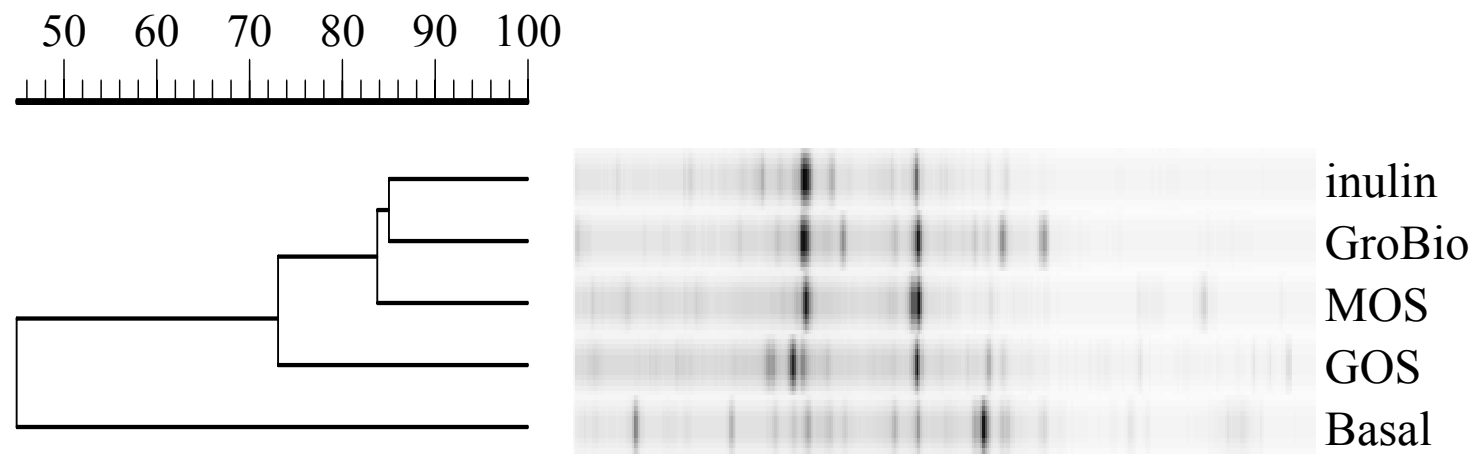


Figure 5. Denaturing gradient gel electrophoresis of bacterial 16S rDNA amplicons from fish intestinal contents. The bar above figure indicates percentage similarity coefficients. Basal = basal diet; GroBio = GroBiotic[®]-A; inulin = inulin; MOS = MOS; GOS = GOS.

different when compared to the communities of fish fed the other prebiotic supplements ($SC < 80\%$). The DGGE patterns from fish fed inulin, GroBiotic[®]-A, and MOS were all related but not identical ($80\% < SC < 95\%$).

Discussion

The addition of prebiotics to the basal did not alter growth performance, FE, or PER of hybrid striped bass as observed in previous studies in which 1% or 2% Grobiotic[®]-A enhanced performance of juvenile and sub-adult fish (Li and Gatlin 2004, 2005). However, in the current experiment the initial fish weight, feeding rate, and length of the trial did not favor maximum growth and might have masked potential differences in fish performance among treatments (Li and Gatlin 2004, 2005). Hybrid striped bass and red drum *Sciaenops ocellatus* fed a diet supplemented with GroBiotic[®]-A in previous experiments generally had greater weight gain compared to fish fed a basal diet (21, 22, unpublished data). Feed efficiency ratios in the current study also were lower than reported for a hybrid striped bass fed a fishmeal based diet supplemented with either 1% or 2% Grobiotic[®]-A (Li and Gatlin 2004, 2005).

Whole-body composition of fish did not vary among treatments in the present study, except for whole-body protein of fish fed Grobiotic[®]-A. Red drum fed a similar diet to that used in the present study had increased protein digestibility when GroBiotic[®]-A was supplemented compared to fish fed a basal diet (Burr et al. 2007). This increased protein digestibility could lead to higher protein deposition in muscle tissue because more protein would be available for deposition after energy needs of the

fish are satisfied. The whole-body ash, moisture, protein and lipid levels were not affected by dietary treatment and are similar to previously reported values (Burr et al. 2006).

This is the first *in vivo* study to use culture-independent techniques to demonstrate that the microbial community of a finfish can be altered with the addition of prebiotics to the diet. Previous studies have examined the effects of inulin on the culturable aerobic/facultative anaerobic bacteria (Mahious et al. 2006, Ringø et al. 2006, Bakke-McKellep et al. 2007). Results of the present study are similar to those reported for rainbow trout *Oncorhynchus mykiss* where the intestinal microbial community was altered, but an increase in fish growth was not detected (Heikkinen et al. 2006).

The observed complexity of the DGGE profiles of hybrid striped bass with two to four dominant bands is different than that reported for haddock larvae *Melanogrammus aeglefinus*, which contained 5-6 dominant bands (Plante et al. 2007). This difference indicates that hybrid striped bass kept in a closed recirculating system had less diversity in the intestinal tract microbial community than haddock larvae maintained in a closed recirculating seawater system. However, the microbial community of the intestinal tract of hybrid striped bass was similar compared to juvenile coho salmon, which had four dominant bands in the DGGE profile (Romero and Navarrete 2006). The addition of inulin to the diet Arctic charr *Salvelinus alpinus* (Ringo et al. 2006) altered the culturable adherent intestinal microbial community. The addition of inulin also increased growth of the turbot and increase *Bacillus* sp. found in the intestinal tract (Mahious et al. 2006). The decreased complexity of the DGGE profiles

when compared to the basal indicates that less genera are present in the intestinal lumen of fish fed inulin, which agrees with studies conducted with Atlantic salmon fed diets supplemented with inulin where diversity of intestinal microorganisms was reduced (Bakke-Mckellep et al. 2007). However, three studies (Mahious et al. 2006, Ringø et al. 2006, Bakke-McKellep et al. 2007) that have looked at the effects of prebiotics on the intestinal microbial community only examined the culturable microbes, ignoring the unculturable microbial community (Ward et al. 1990).

Pond et al. (2006) demonstrated using molecular techniques that the culturable community was different from the unculturable species. The current study also demonstrates that the microbial community cultured in a minimal media varied greatly from the microbial community in the intestinal tract, with the *in vivo* community having greater diversity and not being dominated by a single species as seen in the *in vitro* experiment after 48 h. In order to use an *in vitro* study as a predictor of possible prebiotics, a better media needs to be determined. Media that contains yeast extract, vitamins and fatty acid have been used in other *in vitro* studies with human and dog fecal microbiota (Hughes et al. 2007, Spears et al. 2007).

The current study demonstrated that the four prebiotics were able to alter the intestinal microbiota of the fish *in vivo*, but did not affect the culturable intestinal microbial community. Growth and performance indices of the fish were not affected by the supplementation of the prebiotics. Further studies are needed to identify bacterial species and to determine if disease resistance is increased.

CHAPTER VI

EFFECTS OF PREBIOTICS ON NUTRIENT DIGESTIBILITY OF A SOYBEAN-
MEAL-BASED DIET BY RED DRUM *Sciaenops ocellatus*

Synopsis

Various plant feedstuffs have been evaluated in aquafeeds, but they are generally less nutritious than fish meal, especially for carnivorous fishes. Certain dietary supplements, however, may be able to increase nutrient availability to the host. Prebiotic compounds comprise one such group of supplements and are defined as non-living food ingredients that are selectively metabolized to favor beneficial intestinal bacteria. Among other benefits, those bacteria may enhance nutrient availability to the host organism, as has been observed in some terrestrial animals.

The present study examined the effects of four prebiotics on nutrient and energy digestibility of soybean-meal-based diets by red drum *Sciaenops ocellatus*. The experimental diets were formulated so that approximately 50% of the protein was provided by soybean meal with the remainder from menhaden fish meal. The four prebiotics evaluated were GroBiotic[®]-A, mannanoligosaccharide (MOS), galactooligosaccharide (GOS), and inulin, each added to the basal diet at 1% by weight. A control diet in which all of the protein was provided by fish meal also was evaluated. All diets contained chromium oxide at 1 % by weight, to serve as a non-digestible marker. The digestibility trials were conducted in a recirculating brackish-water (7 to 9 ppt) system with approximately 50-g red drum. Fish were fed the diets for 3 weeks and

then manually stripped to obtain fecal material which was dried and analyzed for energy, protein, lipid, ash, and chromium content. Of all test diets, the fish-meal-based diet had the highest apparent digestibility coefficient (ADC) values— 87% for protein, 87% for lipid, 78% for organic matter and 83% for energy. The diets supplemented with GroBiotic[®]-A, GOS, and MOS had increased protein (82%, 82%, 82%, respectively) and organic-matter digestibility values (69%, 64%, 66%, respectively), compared to the basal diet (69% for protein and 49% for organic matter). Lipid ADC values were decreased in the diets supplemented with inulin, GOS, and MOS (63%, 61%, 61%, respectively), compared to the basal diet (77%). Energy ADC values also were increased in fish fed the GroBiotic-A[®], GOS and MOS diets (73%, 70%, 72%), compared to the basal diet (57%); however, the fish fed the inulin diet had a value of energy ADC (54%) similar to that of fish fed the basal diet. In previous studies, diets supplemented with GroBiotic[®]-A increased growth performance of hybrid striped bass and red drum. Thus, the present data, suggesting that GroBiotic[®]-A enhances nutrient digestibility, provide a plausible explanation for the growth enhancement observed in the previous feeding trials.

Introduction

The use of plant feedstuffs in aquafeeds has received heightened attention in recent years (e.g., Glencross et al. 2005; Gatlin et al. 2007). Soybean meal and other plant feedstuffs have been widely used in diets for omnivorous finfish; however, fish meal is still the major source of protein in prepared feeds for carnivorous finfish species.

Due to various anti-nutritional factors in soybean meal and other plant feedstuffs, the diets of most carnivorous finfish species contain a relatively low percentage of these feedstuffs. In order to increase the amount of plant feedstuffs in the diet of carnivorous species the effects of the various anti-nutritional factors must be mitigated. Probiotics and prebiotics may serve such a role, in that they have been shown to increase digestibility and growth of some finfish species.

Probiotics, potentially beneficial live bacteria in the diet, and prebiotics, non-living food ingredients that are selectively metabolized to favor beneficial gastrointestinal (GI) bacteria, are two types of beneficial diet supplements. The use of probiotics has been studied most extensively in terrestrial vertebrates such as pigs (Sakata et al. 2003; Gardiner et al. 2004), chickens (Patterson and Burkholder 2003) and humans (Fioramonti et al. 2003), but to a more limited extent in fishes (reviewed by Gatesoupe, 1999; Verschuere et al. 2000; Irianto and Austin 2002; Burr et al. 2005). While most studies with probiotics have examined increased disease resistance (Gatesoupe 1999; Irianto and Austin 2002), the potential to increase nutrient availability also exists. In turbot the inclusion of *Vibrio proteolyticus* in the diet has been shown to increase nitrogen retention (De Schrijver and Ollevier 2000).

Increased nutrient digestibility may be due to the microbial community producing enzymes that are either lacking or at low levels in the host. Amylase activity of the GI tract microbiota was examined in five species of fishes: Ayu (sweetfish, *Plecoglossus altivelis*), common carp, channel catfish, Japanese eel and tilapia (Sugita et al. 1997). The study reported that amylase activity occurred in a higher percentage of

the anaerobic bacteria isolated (68.4%) as compared to the isolated aerobes (20%). The greater amylase activity indicated that the anaerobic microbiota of the GI tract may play an important function in the digestive capabilities of the host. Ramirez and Dixon (2003) isolated anaerobes from the GI tract of oscar, angelfish and southern flounder and found most of the bacterial species could be classified as *Clostridium*, *Bacteriodes*, *Porphyromonas*, and *Fusobacterium*. In that study, the enzymatic activity of the bacteria varied greatly, with some enzyme activities present in the microbial community and not endogenous to the host. These authors, however, did not assess microbial processes yielding fermentation products, pathogen inhibition or effects on the digestive capability of the host species (Ramirez and Dixon 2003). Recalcitrant molecules, such as those in dietary fiber, also could become an energy source with enzymatic assistance from the endogenous microbiota. Anaerobic carboxymethylcellulase-producing bacteria have been isolated from the intestinal tract of pinfish (Luzckovich and Stellwag 1993). Such studies indicate that the enzymes produced by the microbiota may assist the host in obtaining energy from otherwise indigestible dietary constituents.

In terrestrial livestock, prebiotics such as oligofructose have been reported to increase bioavailability of glucose and trace elements in the diet (Breves et al. 2001; Bongers and van den Heuvel 2003). Oligofructose also has been shown to increase feed efficiency and weight gain in broiler chickens; whereas, mixed results have been reported for pigs (Flickinger et al. 2003). Such information concerning the effects of prebiotics on nutrient digestibility of fish currently is not available. Therefore, the objective of this study was to determine if prebiotics supplemented in the diet could

increase the digestibility of macronutrients by red drum. A second objective was to determine if a change in the microbial community's genera or species could be detected in fish fed the diets supplemented with various prebiotics.

Methods and Materials

The potential effects of prebiotic supplements on nutrient and energy digestibility were evaluated by conducting a digestibility trial with sub-adult red drum following procedures previously established in this laboratory (Gaylord and Gatlin 1996; Li et al. 2004). The control diet was formulated to contain 40% crude protein, exclusively from fish meal; 10% lipid; and, an estimated available energy of 14.6 kJ/kg (Table 15). Five experimental diets were formulated to be similar to the control diet, but with approximately 50% of the protein supplied by fishmeal and 50% provided by soybean meal. To four of the experimental diets, prebiotics were singularly added at 1% of dry weight in place of cellulose while the basal diet had no prebiotic supplementation. The prebiotics evaluated were GroBiotic[®]-A (consisting of a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products), mannanoligosaccharide (MOS), galacto-oligosaccharide (GOS), and inulin. Chromic

Table 15. Composition (g/100 g dry weight) of each diet.

| | Diet Designation | | | | | | |
|--------------------------------------|------------------|---------|-------|-------------|------|--------|------|
| | IFN | Control | Basal | GroBiotic-A | MOS | inulin | GOS |
| Select Menhaden Meal ¹ | 5-02-009 | 58.7 | 27.1 | 27.1 | 27.1 | 27.1 | 27.1 |
| Soybean Meal, Dehulled | 5-04-612 | n/a | 35.5 | 35.5 | 35.5 | 35.5 | 35.5 |
| Dextrin ⁴ | | 14.7 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 |
| Menhaden Oil ² | 7-08-049 | 6.3 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 |
| Vitamin Premix ³ | | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| Mineral Premix ³ | | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| Carboxymethyl cellulose ⁴ | | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| GroBiotic-A ^{®5} | | n/a | n/a | 1.0 | n/a | n/a | n/a |
| MOS ⁶ | | n/a | n/a | n/a | 1.0 | n/a | n/a |
| inulin ⁷ | | n/a | n/a | n/a | n/a | 1.0 | n/a |
| GOS ⁸ | | n/a | n/a | n/a | n/a | n/a | 1.0 |
| Cellulose ⁴ | | 10.3 | 0.9 | n/a | n/a | n/a | n/a |

Table 15. Continued

| | Diet Designation | | | | | | |
|---------------------------------|------------------|---------|-------|-------------|------|--------|------|
| | IFN | Control | Basal | GroBiotic-A | MOS | inulin | GOS |
| Chromium III Oxide ⁹ | | 1.0 | 1.0 | 0.9 | 0.9 | 0.9 | 0.9 |
| <i>Analyzed Composition</i> (%) | | | | | | | |
| dry weight) ¹⁰ | | | | | | | |
| Protein (%) | | 48.7 | 40.3 | 41.6 | 42.7 | 41.6 | 42.0 |
| Lipid (%) | | 11.7 | 10.7 | 10.0 | 12.6 | 7.9 | 9.6 |
| Gross energy (Kcal/kg) | | 4532 | 4567 | 4668 | 4603 | 4655 | 4635 |

¹ Contained 73.8% protein and 10.0% lipid; Omega Protein Corporation, Inc., Abbeville, AL.

² Omega Protein Corporation, Inc., Reedville, VA

³ Moon and Gatlin (1991)

⁴ USB Corporation, Cleveland, OH

⁵ International Ingredient Corporation, St. Louis, MO

⁶ Alltech Inc., Nicholasville, KY

⁷ Encore Technologies LLC, Plymouth, MN

⁸ Friesland Foods Domo, Zwolle, Netherlands

⁹ Sigma-Aldrich, St. Louis, MO

¹⁰ Means of two analyses

oxide was added to each diet at 1% of dry weight to serve as an inert, indigestible marker. Diets were prepared and stored as previously described (Gaylord and Gatlin 1996).

Fish and culture system

Red drum, *Sciaenops ocellatus*, were contained in a brackish water (8‰) recirculating system comprised of six, 1200-L circular fiberglass tanks, each with approximately 945-L of water and each containing 35 fish weighing approximately 500g each. This six-tank system included a common settling chamber, biological filter and sand filter. Dissolved oxygen was maintained close to air saturation by blowing compressed air through air stones into each tank. Temperature in the system was maintained by conditioning the air to 25°C and a 12:12 light dark cycle was maintained throughout the experiment. Water quality was monitored periodically.

Feeding and fecal collection

The six diets were each assigned to two separate tanks of fish to obtain duplicate fecal samples. All fish were conditioned using a commercial diet (Rangen Extr 400, Buhl, ID) for 2 weeks prior to initiating the digestibility trial. In the first cycle, three of the diets (soybean meal basal, soybean meal basal + 1% Grobiotic[®]-A, and soybean meal basal + 1% inulin) were each fed to fish in two separate tanks for 3 weeks prior to fecal collection to allow the prebiotics to modify the GI tract microbial community. Fish in all tanks were reconditioned on the commercial diet for 2 wk prior to the second cycle

in which the remaining three diets (control, soybean meal basal + 1% MOS, soybean meal basal + 1% GOS) were evaluated. In the second cycle, the tanks of fish that received the soybean meal basal diet were the same as those that received the fishmeal basal diet; thus, these fish were never directly exposed to any of the prebiotics. The other two diets were randomly assigned among the remaining four tanks and all diets were once again fed for 3 weeks. During both cycles, fish were fed to apparent satiation twice daily. On the day of fecal collection, 4 h post feeding, fecal material was collected using the stripping technique (Austreng 1978; Hajen et al. 1993). Fish were physically restrained and pressure was applied to the abdomen to initiate defecation into pans cleaned with 100% ethanol before collection. Fish were returned to their respective tanks and readily recovered from the handling. Samples were collected from all fish in each tank and pooled as one composite sample (averaging 3 g of dried feces) per tank. The fecal material was dried at 60°C for 24 h and placed into sterile bags and stored at -20°C until analysis.

Nutrient analysis of diet and fecal samples

The diets and fecal material were analyzed for organic matter by drying the samples for 2 h at 120°C and then ashing the dry samples at 550°C for 3 h (Association of Official Analytical Chemists, 1990). The dry samples were hydrated and analyzed for lipid content using the chloroform/methanol extraction method (Folch et al., 1957). Crude protein was determined by the Dumas method (Ebeling, 1968) using a Leco Nitrogen Determinator (Model FP-528, Leco Corporation, St. Joseph, MI). The

carbohydrate fraction was estimated by difference as total carbohydrate = [100-(% crude protein + % crude lipid + % ash)]. Energy content was determined using an isoperibol bomb calorimeter (Model 6200, Parr Instrument Company, Boline, IL). Chromic oxide determination was by the method of Furukawa and Tsukahara (1966). Apparent digestibility coefficients were calculated using the standard formula for the indirect method (NRC 1993).

Statistical analysis

Apparent digestibility coefficients for protein, lipid, organic matter and energy in the control and experimental diets were subjected to analysis of variance and Duncan's multiple range test for comparison, using the Statistical Analysis System (SAS, 1985). Statistical significance was set at $P \leq 0.05$.

DNA isolation, PCR

To compare the GI tract microbial populations of fish fed the various diets, genomic DNA was isolated from approximately 0.2 g of dried feces with the Bio-Rad Aqua Pure DNA Isolation Kit (Bio-Rad, Hercules, CA) using the method supplied by the manufacturer, with the following modifications. The pellets were suspended in 800 μ l of the DNA lysis buffer. Twenty μ l of 20 mg/ml lysozyme were added and mixed with a sterile pestel. The solution was then incubated at 37°C for 2 h, centrifuged at 20,800 x *g* for 3 min and the supernatant removed and placed into a clean 1.5-ml microcentrifuge

tube. A 1.5 µl aliquot of the RNase (4 mg/ml) solution was subsequently added and the mixture was incubated at 37°C for 45 min.

PCR was conducted using the method of Hume et al. (2003) with bacteria-specific PCR primers to conserved regions flanking the variable V3 region of 16S rDNA. The primers (50 pmol of each primer; primer 2, 5'-ATTACC GCGGCTGCTGG-3'; primer 3 with a 40 base pair GC clamp (Muyzer et al., 1993) 5'-CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG GCAGCAG-3') were mixed with Jump Start Red-Taq Ready Mix (Sigma Chemical Company, St. Louis, MO) according to the manufacturer's instructions, 250 ng of pooled (83 ng/replicate) template DNA was added along with 10 µg of bovine serum albumin (BSA) to help stabilize the reaction. The PCR amplifications were performed on a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). A touchdown PCR program was used to minimize artificial by-products. The program used was as follows: 1) denaturation at 94.9°C for 2 min; 2) denaturation at 94.0°C for 1 min; 3) annealing at 67°C for 45 s, -0.5°C per cycle (to minimize formation of artificial products) (Hume et al., 2003); 4) extension at 72°C for 2 min; repeat steps 2 to 4 for 17 cycles; 5) denaturation at 94°C for 1 min; 6) annealing at 58°C for 45 sec; 7) repeat steps 6 to 7 for 12 cycles; 8) extension at 72°C for 30 min; 9) 4°C final.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was run following the method of Hume et al. (2003) as modified from Muyzer et al. (1993). The amplicons were

separated on 8 % polyacrylamide gels [(vol/vol) acrylamide-bisacrylamide ratio of 37.5:1 (Bio-Rad, Richmond, CA)] with a 30 to 60% urea-formamide gradient (100% denaturing 7M urea and 40% formamide) using a Dcode System (Bio-Rad, Hercules, CA). The amplicons were mixed with an equal volume of 2X loading buffer [0.05% (wt/vol) bromophenol blue; 0.05% (wt/vol) xylene cyanol; and 70% (vol/vol) glycerol] and 7 µl was loaded into each sample well (16-well comb). The gels were run at 60 volts for 17 hours in 0.5X TAE (20 mM Tris (pH 7.4); 10 mM sodium acetate; 0.5 M EDTA; Bio-Rad, Hercules, CA) at 59°C. Gels were stained for 30 min with SYBR Green I (USA Amersham Life Sciences, Cleveland, OH) diluted 1:10,000. The fragment analysis pattern relatedness was determined with Molecular Analysis Fingerprinting software (v 1.6; Bio-Rad, Hercules, CA). This analysis is based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages (UPGMA) for clustering. Comparisons between sample band patterns were expressed as a percentage similarity coefficient (%SC).

Results

The crude protein apparent digestibility coefficient (ADC) values ranged from 69.2 to 87.0 %, with the fish-meal control diet having the highest protein digestibility and the basal experimental diet having the lowest digestibility (Table 16). GroBiotic[®]-A, MOS and GOS supplementation all significantly increased crude protein ADC values of the experimental diets; whereas, inulin did not promote a significant increase. Total

Table 16. Percent apparent digestibility coefficient (ADC) values for red drum fed the fish meal (FM) control diet or soybean meal (SBM) based diets either unsupplemented (basal) or supplemented with either GroBiotic[®]-A, inulin, GOS or MOS at 1% by weight¹

| | Protein | | Organic | Energy | CHO ² |
|----------------|--------------------------|---------------------------|--------------------------|--------------------------|----------------------------|
| | ADC | Lipid ADC | matter | ADC | ADC |
| | | | ADC | | |
| FM Control | 86.96 ^A (0.6) | 87.42 ^A (0.3) | 78.42 ^A (0.6) | 82.64 ^A (0.9) | 56.08 ^A (0.74) |
| SBM Basal | 69.19 ^B (3.0) | 76.81 ^B (2.3) | 49.15 ^C (4.1) | 57.03 ^B (3.2) | 19.13 ^C (8.9) |
| 1% GroBiotic-A | 82.38 ^A (1.3) | 81.97 ^{AB} (0.3) | 68.76 ^B (2.2) | 73.43 ^A (1.5) | 50.16 ^A (5.5) |
| 1% Inulin | 74.09 ^B (3.5) | 63.49 ^C (7.5) | 54.57 ^C (6.0) | 53.91 ^B (9.1) | 32.6 ^{BC} (1.5) |
| 1% GOS | 81.75 ^A (1.6) | 61.40 ^C (3.5) | 63.60 ^B (3.6) | 70.40 ^A (2.1) | 43.55 ^{AB} (11.1) |
| 1% MOS | 81.57 ^A (0.9) | 61.30 ^C (3.0) | 66.31 ^B (2.4) | 72.30 ^A (2.1) | 48.58 ^A (0.42) |
| | <i>P</i> = 0.0019 | <i>P</i> = 0.0007 | <i>P</i> = 0.0015 | <i>P</i> = 0.0102 | <i>P</i> = 0.0005 |

¹Mean of two replicates (\pm standard error of mean). Values within columns and with a common superscript letter do not differ significantly ($P > 0.05$)

²Determined by difference

lipid digestibility was significantly higher in the control diet than for all other diets except the one supplemented with 1% GroBiotic[®]-A. Lipid digestibility values ranged from a high of 87.4% to a low of 61.3% (Table 16). The diet supplemented with 1% GroBiotic[®]-A had significantly higher lipid ADC than the diets containing the other prebiotics, but did not differ significantly from the basal diet ($P \leq 0.05$). The carbohydrate ADC values ranged from 19.1 to 56.1%, with the fish meal control having the highest value. The diets supplemented with GroBiotic[®]-A, MOS and GOS all had significantly higher carbohydrate ADC values compared to the soybean meal basal diet ($P \leq 0.05$). Organic matter digestibility coefficients ranged from 78.4% for the control diet to 49.2% for the soybean meal basal diet. All of the prebiotics except inulin resulted in significantly increased organic matter ADC values when compared to the soybean meal basal diet. Inulin did show a trend for increased organic matter digestibility; however, due to within-treatment variation, this increase was not significant.

The ADC values for energy ranged from 82.6% for the control diet to 53.9% for the inulin-supplemented diet. The diets supplemented with GroBiotic[®]-A, GOS and MOS all had higher energy digestibility (73.4, 70.4, and 72.3%, respectively) compared to the basal diet and were not statistically different from the control.

The dendrogram analysis of microbial gene products from fish fed the various diets showed great variation among tanks ($SC < 80\%$) (Fig. 6). The microbiota from the GI tract of fish in tanks 18 and 17 fed the inulin and GroBiotic[®]-A diets were almost identical ($SC > 95\%$), but were not related to microbiota ($SC < 36\%$) from fish in tanks

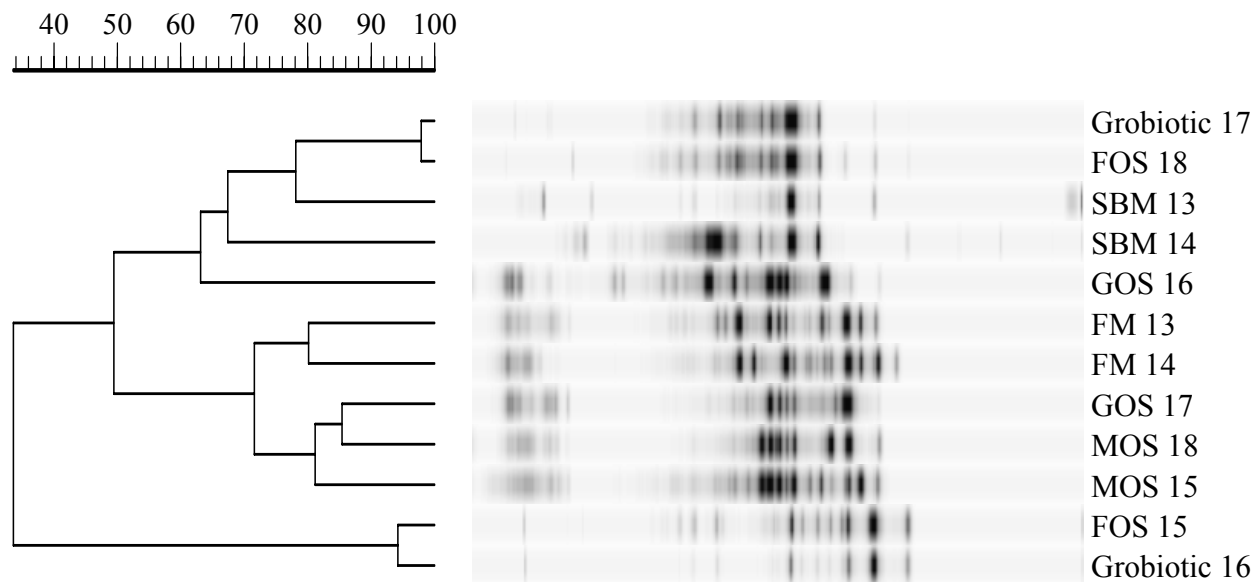


Fig. 6. Denaturing gradient gel electrophoresis of bacterial 16S rDNA amplicons from red drum fecal contents. FM = control diet, SBM = basal diet, Grobionic[®]-A = SBM + 1% Grobionic[®]-A, inulin = SBM + 1% inulin, MOS = SBM + 1% MOS, and GOS = SBM + 1% GOS. The number indicates the tank number. The bar above figure indicates percentage similarity coefficients.

16 and 15 (also with SC > 95%), which were fed the same diets. The microbial communities from fish fed OS and the basal diet were related, but the patterns of GOS-fed fish was more closely related to one of the MOS-fed groups than that of the other group fed the GOS-supplemented diet. The dendrogram indicates that the microbial populations were varied in the tanks and unique microbial populations may have existed in each tank even though the water was recirculating to all tanks.

Discussion

This is the first study that has considered the effects of prebiotics on the digestibility of macronutrients in fish. The results of this study reveal that prebiotics can increase the digestibility of macronutrients in a soybean-meal/fishmeal-based diet. The fishmeal control diet had the best apparent digestibility coefficients for all of the nutrients examined. These results are supported by the findings of Gaylord and Gatlin (1996) and Li et al. (2004) in which fishmeals had the best digestibility coefficients of any tested ingredients for red drum. The apparent protein digestibility values for the fishmeal diet in the present study are similar to those reported for other species such as yellowfin seabream (Wu et al. 2006), rainbow trout (Glencross et al. 2005; Ogunkoya et al. 2006), Atlantic salmon (Refstie et al. 2000), greenback flounder (Bransden and Carter 1999) and channel catfish (Wilson and Poe 1985).

The apparent digestibility coefficient for protein in the prebiotic-supplemented diets, with the exception of inulin-supplemented diet, was significantly higher when compared to the basal diet and did not differ significantly from the control diet. These

results indicate GroBiotic[®]-A, MOS and GOS apparently mitigated some of the anti-nutritional factors present in soybean meal, although the specific mechanism was not determined. The soybean meal was included at 35% in the experimental diets, which is relatively high compared to inclusions levels in diets for rainbow trout and Atlantic salmon (Refstie et al. 2000; 2001).

The apparent digestibility of lipid by red drum decreased by an average of 14.7% with the inclusion of GOS, MOS and inulin, but increased with the supplementation of GroBiotic[®]-A. The decrease in lipid uptake could have been due to the energy needs of the host being met by the catabolism of carbohydrates and protein. Another possibility is that the prebiotics interfered with the uptake of dietary lipids by down-regulating other enzymes involved in lipid digestion/absorption. Further study is needed to determine the mode of action for this reduction in lipid digestibility.

Energy and organic matter ADC values were significantly increased with the inclusion of GroBiotic[®]-A, MOS and GOS; but, the fishmeal control diet again had the highest energy and organic matter ADC values compared to the diets containing soybean meal, which result is similar to that observed with rainbow trout, Atlantic salmon, red drum, and catfish, (Wilson and Poe 1985; Bransden and Carter 1999; Refstie et al. 2000; Glencross et al. 2005; Ogunkoya et al. 2006). Data from this experiment indicated prebiotics made more of the nutrients in soybean meal accessible for the animal, either through mitigation of the anti-nutritional factors or through a microbially mediated pathway. Further study is needed to elucidate the mechanisms responsible for the increased ADC values observed in this study.

The detectable microbial community isolated from the fecal matter of fish fed the different diets varied among treatments and among tanks without any discernable pattern. Only the microbiota from red drum fed the control diet clustered as most related; however, even these two communities were not very closely related (<90% SC). The communities were complex and did not have a single species that dominated, as was evident in an *in vitro* experiment with red drum (unpublished data). The diet did influence the microbial community as the fish fed the control diet had a distinct community when compared to fish fed the experimental diets. The effects of a soybean-meal-based diet on the intestinal microbial community recently were reported for Atlantic cod (Refstie et al. 2006) and rainbow trout (Heikkinen et al. 2006). The culturable microbiota were determined to be distinct when soybean meal was included in the cod diet when compared to a diet containing fishmeal as the only protein source (Refstie et al. 2006). The culturable intestinal bacteria were less abundant in rainbow trout fed a diet containing soybean meal when compared to a fish fed a fishmeal diet (Heikkinen et al. 2006).

The varied GI tract microbial communities are possible because the fecal material microbial community might be different from the microbial community in the intestinal tract, both the attached and luminal microbes (Moore et al. 1974). Another possibility for the similarity of the microbial community in fish fed the different experimental diets is that the same microorganisms might be present in the GI tract but utilize different biochemical pathways in fish fed the basal diet when compared to

prebiotic-supplemented diets. Further study is needed to elucidate the reasons for these differences.

In conclusion, all of the prebiotics evaluated increased protein digestibility in the experimental diets. The fishmeal control diet had the highest digestibility, with the GroBiotic[®]-A-supplemented diet having similar nutrient digestibility coefficients. The microbial communities varied among tanks without any discernable pattern attributed to the prebiotics. Further study is needed to determine the mechanisms that altered the protein, lipid, organic matter, and lipid digestibility of the prebiotic-supplemented diets.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Prebiotics have received much attention as a food additive for terrestrial livestock, especially as a means of controlling disease and increasing growth. Interest in use of prebiotics is expanding because antibiotics increasingly are being banned from production of food animals, and alternatives are being sought to take their place.

The studies presented in this dissertation represent the first effort to comprehensively evaluate prebiotics in two important fish species. In the *in vitro* studies, Grobiotic[®]-A and brewers yeast altered the culturable intestinal microbial community of red drum, but the addition of prebiotics did not affect the culturable intestinal microbial community of hybrid striped bass. The culturable microbial community of red drum had greater diversity than the microbial community of hybrid striped bass. The culturable microbial community of red drum had two major bands, *Lactococcus lactis* and *Aeromonas* sp., while hybrid striped bass had only a single band, *Fusobacteria bacterium*. Volatile fatty acid production was not altered by the addition of prebiotics to the diet in either of two experiments. However, in *in vivo* studies, the intestinal microbiota of hybrid striped bass was altered with the addition of GroBiotic[®]-A, inulin, MOS and GOS but the intestinal microbial community of red drum was unaltered. The intestinal microbial community of hybrid striped bass was also more complex than the culturable microbial community, while the intestinal microbial community of red drum was much simpler. Increased growth was not detected in either *in vivo* experiment. At present, the *in vitro* experiments conducted with fish do not

appear to be good indicators of prebiotic potential in the intestine of the host. This could be due to diversity in the microbes present in the intestine, or possibly is because the feed additive is entirely absorbed by the host and thus does not act as a prebiotic.

In the digestibility experiment with red drum, all of the prebiotic-supplemented diets except the one with inulin increased protein and organic matter digestibility. The digestibility of lipid decreased with all of the diets except for the GroBiotic[®]-A-supplemented diet. The soybean meal/fish meal diet supplemented with GroBiotic[®]-A was not distinguishable from a fishmeal-based diet in terms of nutrient digestibility. The microbial communities did not show any pattern when compared to the basal diet, indicating that the microbes may have altered certain metabolic pathways or that the prebiotics were interacting with the host on a molecular level. Thus, these various experiments with prebiotics indicated that the intestinal microbiota *in vivo* could be altered, but there was not any detectable increase in performance. Further studies are needed to determine the effects on disease resistance and long-term performance compared to a basal diet.

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